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THE RATE OF INACTIVATION BY ULTRA-VIOLET IRRADIATION AS A MEANS OF DISTINGUISHING ANTIGENICALLY DIFFERENT STRAINS OF AFRICAN HORSE-SICKNESS VIRUS.

A. POLSON AND JOAN DENT.

From the Section of Biophysics, Onderstepoort, South Africa.

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A PLURALITY of antigenically different strains of African horse-sickness virus has long been accepted, but was proved conclusively by cross-immunity tests in horses and by the results of intracerebral protection tests in mice (Alexander, 1935). In all approximately 25 different strains of virus have been isolated, the antigenic variation being based largely upon the mouse protection test (Alexander, personal communication). The differentiation of so large a number of antigenic variants by this method is both laborious and time-consuming, so that the elaboration of a simpler and more rapid method is highly desirable. It is submitted that the rate of inactivation by ultra-violet irradiation provides such a means of differentiation. As yet the method has been applied only to a limited number of neurotropically adapted strains of virus. Application of the method to a larger number of strains and also to other viruses appears to be indicated.

It may be pointed out that particle size determination by ultracentfriugation and ultrafiltration showed that six different strains have the same particle size of approximately 50 mμ (Polson, 1941*a*). Electrophoresis experiments also failed to show any difference in isoelectric points or mobilities amongst several strains of the virus (Polson, 1941*b*).

MATERIALS AND METHOD.

The experimental work involved was performed under identical conditions for all the strains investigated. As the standard virus diluent, i.e. 10 per cent horse serum in saline, was unsuitable as a virus dispersion medium on account of its high ultraviolet light absorbing properties, it was not used in the present work. Instead the dialysate of one part of horse serum against 3 parts of saline was used ; this was found equally effective for preserving the virus.

Eight infected brains of homogeneously bred mice of the age-group 5 to 6 weeks, killed *in extremis* were emulsified in 80 c.c. of serum dialysate and the emulsion clarified by centrifugation in a Clay-Adams angle centrifuge spun at 3000 r.p.m. for one hour. Additional clarification by ultra-filtration through gradocol membranes had no effect on the rate of inactivation of the virus and was avoided as the titres of the virus emulsions may be reduced.

The inactivation of the virus was done in quartz tubes of internal diameter 10 mm. and wall thickness 2 mm. These tubes were rotated individually under a source of ultra-violet radiation placed exactly 25 cm. distant in the manner shown in Fig. 1. The ultra-violet radiation was supplied by a Standard Hanovia lamp with Arc Tube No. S.B. 15437, operated on supply of 250 volt A.C. No attempts were made to select particular wavelengths for the inactivation. With this lamp placed at such a distance from the virus emulsion the heating effect was negligible and no special precautions were taken to eliminate it.

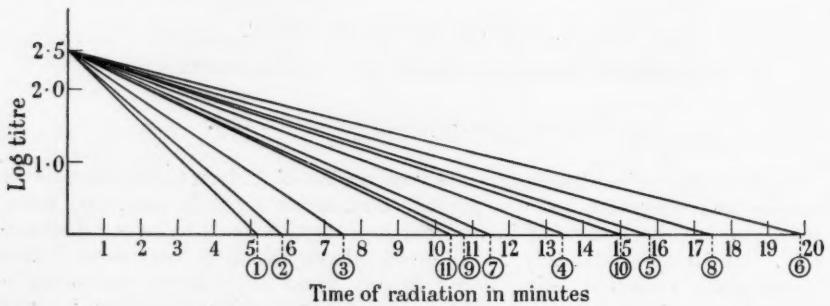

FIG. 2.—Inactivation curves for the strains : (1) Pirie, (2) Galpin, (3) 1180, (4) 114, (5) O.D., (6) 1178, (7) 409, (8) Vryheid, (9) 501, (10) Palestine, and (11) Westermann. The log of the titre is plotted against the time of radiation in minutes.

The estimation of virus activity was done by the intracerebral injection of serial two-fold dilutions of the irradiated fluids in mice.

The following strains were investigated : 1180, 1178, Vryheid O.D. 114, 409, 501, Galpin, Pirie, Westermann, Palestine, L, N.M. 865 and Cedara.

For lack of a rational scheme of classification the symbols used for labelling the different strains were derived from an abbreviation of the laboratory number of the horses that were originally injected with the different strains or from the locality where the strains made their first appearance.

RESULTS.

The results of these irradiation experiments are best presented in graphical form, in which the logarithm of the titre of the surviving virus is plotted against the dosage of ultra-violet radiation or, with constant illumination, as was used in these experiments, as a function of the time of irradiation. In Fig. 2 are given the results of such experiments on different strains of the virus.

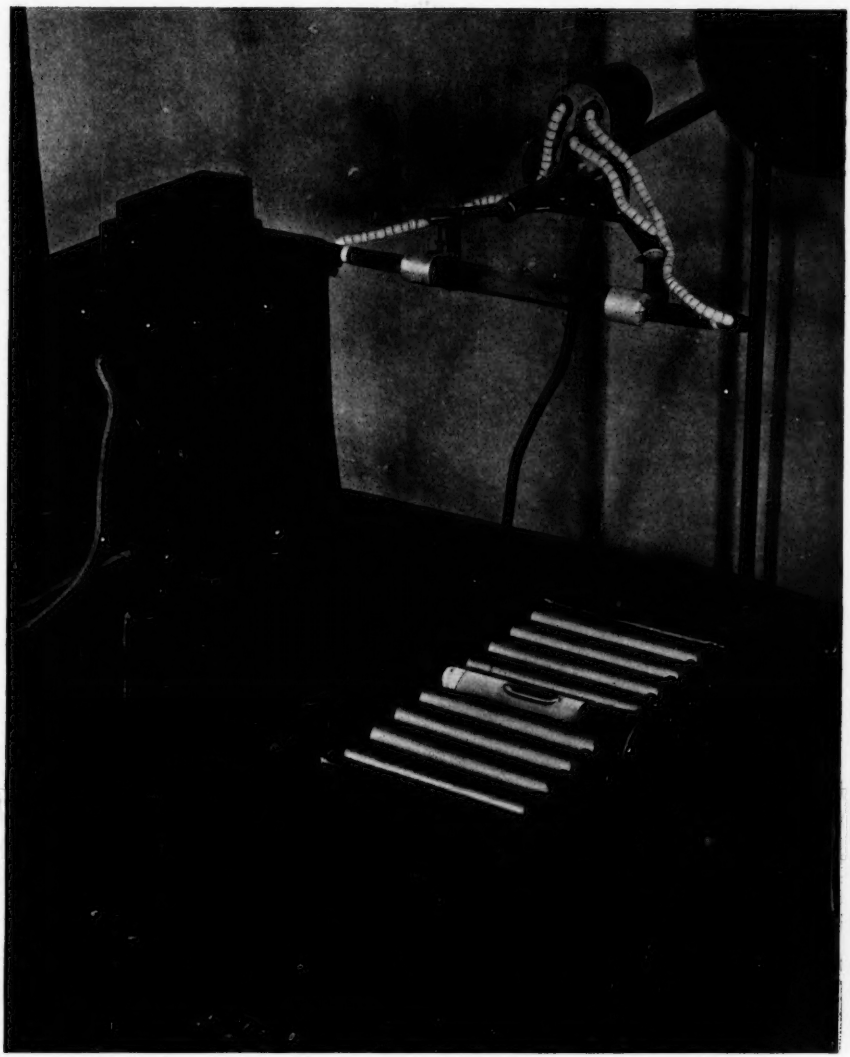

FIG. 1.—Apparatus used for the ultra-violet inactivation studies. The apparatus was constructed by Dr. W. O. Neitz of this laboratory.

In Table I are given the rates of inactivation expressed as d log T/dt. The symbols T and t represent the titre and time of irradiation respectively. This factor is constant for a particular strain.

TABLE I.—*Rates of Inactivation of Different Strains of African Horse-sickness Virus.*

Source of illumination, Standard Hanovia Lamp Arc tube S.B. 15437. Voltage Supply 250 volt A.C. Distance 25 cm.; dispersion medium = dialysate of 1 part horse serum against 3 parts saline.

Strain.	d Log T/dt.
Pirie	0·481
Galpin	0·423
1180	0·333
Westermann	0·240
501	0·234
L	0·227
409	0·217
N.M. 865	0·208
114	0·186
Cedara	0·182
Palestine	0·166
O.D.	0·159
Vryheid	0·143
1178	0·125

DISCUSSION.

Consideration of the data in Table I and the curves in Fig. 2 shows that there is a considerable difference in the rates of inactivation of different strains of virus which are immunologically dissimilar. This, in itself, is remarkable since it has been pointed out that by particle size determination and by electrophoresis no differences have been noted. It is interesting to speculate whether similar rates of inactivation are correlated in any way with similarity of antigenic structure. Data on which to form a definite opinion on this point are at present inadequate. At this institute some 25 antigenically different strains of horse-sickness virus have been isolated and are being examined critically by *in vivo* cross-immunity tests and by *in vitro* serum virus neutralization tests. This work is as yet incomplete, but it is worthy of note that Strains Pirie and Galpin have been found to be antigenically identical. Similarly, Strains Palestine and O.D. bear a marked resemblance, whereas the two pairs are widely dissimilar. It would appear that if the rate of inactivation by ultra-violet light cannot be used to establish the identity of different virus strains at least markedly different rates are correlated with divergent antigenic structure.

It would be of the greatest interest to determine whether strains of foot and mouth virus or equine encephalomyelitis could be differentiated by application of this technique.

The magnitude of the error involved in the determination of the rate of inactivation can be estimated at approximately 10 per cent, judging from the differences in the rates at which identical strains are inactivated.

SUMMARY.

Inactivation by ultra-violet radiation offers a means of differentiating strains of horse-sickness virus.

Strains that are antigenically identical show similar rates of inactivation.

In conclusion we want to express our gratitude to Dr. R. A. Alexander for his interest in this work, and to the Director, Dr. Gilles de Kock, for enabling this work to be carried out.

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THE PATHOGENESIS OF TUBERCULOSIS IN MICE INFECTED INTRAVENOUSLY WITH HUMAN TUBERCLE BACILLI; THE USE OF MICE IN CHEMOTHERAPEUTIC TESTS.

G. T. STEWART.

Imperial Chemical Industries Limited, Biological Laboratories, Hexagon House, Manchester, 9.

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It is clear from the literature, reviewed recently by Raleigh and Youmans (1948*a*), that white mice can be easily infected with mammalian and avian varieties of *M. tuberculosis* by several routes. The type of disease produced varies according to the variety, virulence and dose of the bacillus, and the route of inoculation. There are major discrepancies, however, between the observations of different workers, especially with regard to the virulence of the organism, the immune-response of the mouse and the exact histogenesis of the lesions.

Discrepancies arising from variations in dose appear to have been largely overcome in recent work by the use of weighed inocula. Variations in virulence of different strains of mammalian tubercle bacilli are still imperfectly understood. Thus Schwabacher and Wilson (1937) found no difference in mouse-virulence between certain human and bovine strains, whereas Stamatin and Stamatin (1939) found the bovine strain to be about ten times as virulent as the human strain. Little or no evidence is available about differences in behaviour between different members and different doses of any one (so-called) strain. Alterations in virulence may well arise in respect of ages of the cultures, medium of growth, species of mouse (Gerstle and Thomas, 1941) and other factors; it is obvious that the term virulence cannot at present be assigned an absolute scientific value, and it is therefore important, especially in chemotherapeutic tests with murine tuberculosis, for each investigator to recognize any variations in the experimental infection employed.

The researches of Schwabacher and Wilson (1937) showed that murine tuberculosis was essentially a progressive disease. Inocula of 10^6 to 10^8 mammalian bacilli, injected intravenously into mice, caused death with specific lesions in 4 weeks to 4 months; smaller inocula, 10^2 to 10^5 bacilli, induced a chronic but still progressive disease. For the induction of a still more chronic infection these workers recommended the use of avirulent strains, or raising the resistance of the mice by preliminary vaccination. They conceded, nevertheless, that neither procedure gave a disease strictly comparable to tuberculosis in man.

The use of intravenously-infected mice in the experimental chemotherapy of tuberculosis has been described by Martin (1946) and Raleigh and Youmans (1948*b*). Martin assessed the activity of a drug by prolongation of survival-time in treated groups of mice. This single criterion was criticized by Raleigh and Youmans, who made a detailed study of the histopathological evolution of the

experimental disease and formulated from this a more complex system of assessment.

The present investigation was made to define the pathogenesis of the standard infection used in these laboratories, to study some of the variable factors described above, and to correlate the pathological findings with weight and mortality. A scheme of histopathological assessment, less complex than that of Raleigh and Youmans, was devised for application to chemotherapeutic tests.

METHODS.

Infection in mice.

Groups of 20 to 30 white mice were inoculated intravenously with known weights of *M. tuberculosis*, harvested from 17-day cultures grown on Lowenstein's medium. The standard infection, used routinely in these laboratories, is induced by injecting 0·75 mg. Strain 905 in 0·1 c.c. distilled water, as described by Martin (1946). For purposes of comparison, infections with different doses of this and other human strains of *M. tuberculosis* were also studied, as described below.

Assessment of results.

The mice were weighed at weekly intervals, or oftener, and deaths were recorded daily. Each dead animal was examined for the presence of specific lesions. Tissues for histological examination were taken as follows from :—

(i) Mice killed at random, in groups of at least three, from the various infected groups at stated intervals after inoculation.

(ii) Selected mice which were more severely ill than other members of the group.

(iii) Mice which had recently died, and in which post-mortem decomposition was not advanced.

TABLE I.—*Pathological Assessment of Lung Changes in Mice Infected with* M. tuberculosis.

Score.	Macroscopic changes.	Histology.	*M. tuberculosis.*
0	Normal	Normal or slightly congested	Absent.
1	Congested (all degrees) or oedematous (f r o t h y): No obvious nodules.	Congested. Alveolar haemorrhages. Increase in mononuclears and polynuclears. Thickening of septa	Scanty intracellular.
2	A few grey, pin-head nodules. Remainder of lung normal or congested	Focal proliferative lesions, affecting 10–25 per cent of section. No necrosis	Solitary, or 4–5 clumped in macrophages of proliferative foci.
3	Large yellow nodules and consolidation throughout lungs	Necrotic lesions affecting 25–50 per cent of section ; *or* proliferative lesions affecting >50 per cent of section ; *or* consolidation, with alveolar breakdown, affecting entire section	Small clumps of 6–12 throughout lung.
4	—	Necrotic lesions affecting > 50 per cent of section	Masses.

The lung of each mouse is assigned a score for each of the three columns in the table; the total scores for each mouse are then added, and divided by the number of mice examined, to give an index of the severity of the lesions in mice of that group.

The lungs from these mice were removed *en bloc*; the macroscopic changes in the lungs and other organs were noted, and representative portions transferred to Zenker-acetic for histological sections. Staining was by Ziehl-Neelsen and haematoxylin-eosin.

Weight and mortality data for each type of infection were derived from independent groups, in which none of the mice were sacrificed. Dead mice were examined for the presence of tuberculous lesions, and non-specific deaths were excluded from the records.

The gross and microscopic changes in the lungs were assessed according to the scheme shown in Table I.

RESULTS.

Infection with virulent human tubercle bacilli.

Inoculum of 0·75 mg. Strain 905.

In a typical experiment, intravenous inoculation of 0·75 mg. Strain 905 caused loss of weight and death of all the mice in the group between 20 and 33

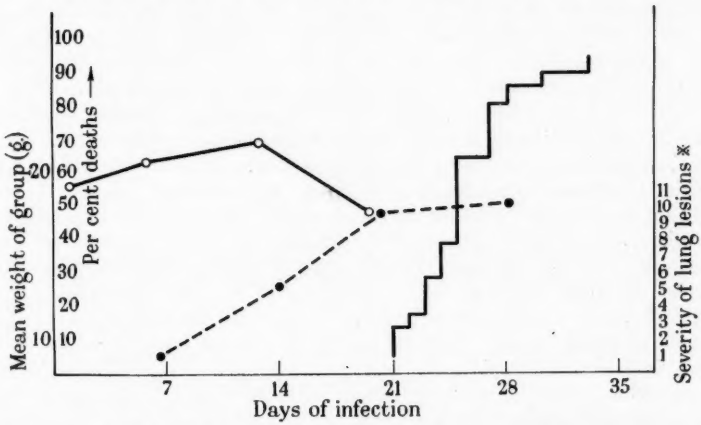

FIG. 1.—Course of infection in mice inoculated with 0·75 mg. *M. tuberculosis*, Strain 905.

Weight o——o

Deaths % ⌐¹

Lesions ●——●

* Mean value scored as in Table I.

Lung	Gross changes	Normal or congested	Grey nodules, congestion, œdema	Yellow nodules, consolidation.
	Histology	Diffuse cellular infiltration	Proliferative foci	Necrotic lesions.
	Bacteria	Scanty, intracellular	Numerous small clumps, intracellular	Masses.
	Liver	Normal	Small cellular foci	Cellular or necrotic foci.
	Spleen	,,	Normal	Enlarged lympho-reticular proliferation.
	Days	0–7	7–14	14–33.

days, the mean survival time being 24·1 days (S.D. 5·1). One non-specific death occurred before 20 days; the remainder of the mice died with nodular lesions in the lungs, containing innumerable acid-fast bacilli. The mice gained weight during the first 14 days of the infection; thereafter loss of weight and illness developed rapidly (Fig. 1).

Lungs, examined at 4 hours and 24 hours after inoculation, appeared slightly congested; isolated or small clumps of acid-fast bacilli were readily found. Microscopically the septa showed diffuse infiltration with mononuclear and polymorphonuclear cells. Between the 2nd and 7th day after inoculation acid-fast bacilli were difficult to find, and no further pathological changes were observed in lung sections. Between the 7th and 14th days minute grey nodules could be discerned on the lung-surfaces, with a variable degree of congestion, cyanosis or oedematous enlargement of the lobes. Histologically, the alveolar septa were thickened by vascular congestion and mononuclear infiltration; in places the mononuclear cells were aggregated into foci, infiltrating or replacing many alveolar compartments and forming the earliest nodules. These foci were essentially proliferative, with no areas of necrosis. Single, or 6 to 12 clumped acid-fast bacilli could be seen within the mononuclears; frequently swollen or fragmented clusters of brown, acid-fast and eosinophilic material were observed intracellularly, and presumably represented abnormal or partially-digested bacilli. These proliferative foci were usually perivascular in distribution, and not, at this stage, extensive; the remainder of the lung tissue showed small haemorrhages, septal thickening and acidophilic exudate into the alveoli (Fig. 2).

No definite lesions were observed in other organs during the first 14 days of the infection. Thereafter, minute grey foci were observed in the liver; these corresponded histologically to cellular areas containing a few intracellular acid-fast bacilli. Splenomegaly, due to lymphoid and mononuclear proliferation, also occurred after 14 days, but acid-fact bacilli were excessively rare.

Between the 14th and 18th days the proliferative lesions expanded and the lungs became studded with yellow or greyish nodules, the intervening areas being cyanosed and consolidated. Histologically the nodules were composed of dense aggregates in the alveoli of mono- and polynuclear cells, and acid-fast bacilli; the alveolar septa between these cellular foci were either destroyed, or persisted only as thready remnants (Fig. 3 and 5).

Until about the 18th day the necrotic element in the histological picture was limited, and the bulk of the lesion was formed by confluent cellular foci affecting up to half of the area of the section. The remainder of the lung showed diffuse cellular infiltration, septal thickening and alveolar exudates. The lesions contained innumerable clumps of acid-fast bacilli, and isolated bacilli were present throughout the lung. After the 18th day, the lesions became largely necrotic with widespread alveolar breakdown and rapid multiplication of the bacteria, forming massive acid-fast blocks visible to the naked-eye in stained specimens (Fig. 4 and 6).

Liver lesions and splenomegaly were conspicuous after the 21st day; the former consisted of cellular and necrotic foci containing scanty acid-fast bacilli, but the splenic enlargement was attributable to general cellular hyperplasia, without localized lesions. Minute cellular foci were noted in some kidney sections, but there was no evidence of widespread extra-pulmonary tuberculosis.

Mononuclears were the predominant cells in the lung lesions. In the early

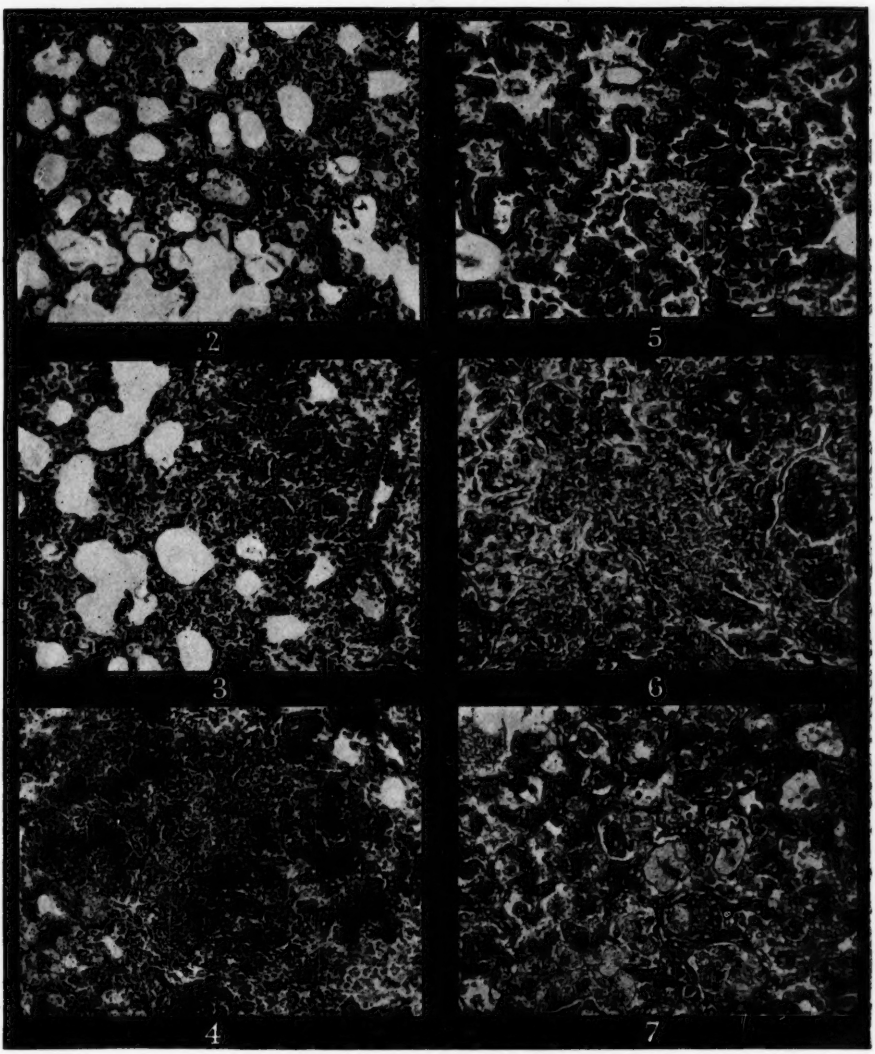

FIG. 2–6.—Lungs of mice infected with 0·75 mg. *M. tuberculosis* Strain 905.
FIG. 2.—7th day of infection. Infiltration of septa and alveoli by inflammatory cells. × 90.
FIG. 3 and 5.—14th day of infection. Proliferative lesions. × 90 and × 250.
FIG. 4 and 6.—21st day of infection. Necrotic lesions. × 90 and × 250.
FIG. 7.—Lung of mouse infected with 0·75 mg. *M. tuberculosis* Strain M, 60th day of infection. Foam-cell reaction in alveoli. × 250.

stages (microscopic focal lesions) these cells were large and epithelioid in type; the cytoplasm was sometimes foamy, or it might contain bacilli showing normal or altered staining reactions, as described above. As the infection advanced, small mononuclears and lymphocytes became more numerous. Polymorphonuclear cells were noted mainly in the earliest stage of interstitial inflammation and, at the other extreme, around the necrotic areas. Giant cells and fibroblasts were absent throughout; no true tubercles were formed; fibrosis and cavitation did not occur.

Inoculum of 0·02 mg. Strain 905.

Intravenous inoculation with this dose caused illness and death of all the mice in the group between 25 and 100 days. Fifty per cent of the group died between the 25th and 66th days (mean 33·5). On average, the mice gained weight until the 30th day of infection and thereafter declined, though a few mice surviving for long periods continued to gain weight.

During the first 14 days isolated bacilli could be found in the lungs, but no specific morbid reaction occurred. At 14 days small proliferative foci appeared and the bacilli multiplied. Large proliferative foci and early necrotic changes were evident in some mice at 21 days. With this infection survival-time varied considerably. Mice dying early (21 days) showed necrotic lesions, whereas those surviving for longer than 35 days seemed able to arrest the disease at the stage of cell-proliferation, and even gained weight in the later stages of the infection.

Inoculum of 0·0004–0·01 mg. Strain 905.

The survival time of mice lengthened and the severity of the lesions decreased as the infecting dose of *M. tuberculosis* was lowered (Table II). With doses of 0·01 mg. and below, the lesions observed at 70 days were mainly or entirely proliferative. The infection was, nevertheless, progressive and unremitting. A conspicuous "foam-cell" reaction was often observed in the pulmonary alveoli (Fig. 7). Splenomegaly, without focal lesions, was again observed in the later stages of infection, as with inocula of 0·75 mg. and 0·02 mg., but lesions were minimal or absent in the other viscera.

TABLE II.—*Effect of Different Inocula of* M. tuberculosis *Strain* 905 *in Mice.*

Inoculum. (mg.).	Specific deaths (days).		Severity of lesions at (days).*
	Minimum.	Maximum.	
0·0004	> 100	—	4·3 (70)
0·002	70–100	—	6·2 (70)
0·01	< 70	—	8·2 (70)
0·02	21	100	10 (35)
0·75	17	33	11 (21)

* Mean of 5 mice, assessed as in Table I.

Infection with tubercle bacilli of low virulence.

When mice were inoculated intravenously with 0·75 mg. of Strain M, deaths with specific lesions began to occur 27 days after infection; the mean survival time was 45·9 days (S.D. 14·9), and one mouse survived for 102 days. The pathological changes followed the pattern of slow evolution described above for small

inocula of virulent organisms. Ultimately there was massive pulmonary involvement, but the lesions, even at a later stage, were characterized by proliferative rather than necrotic changes; the alveoli contained many foam cells; acid-fast bacilli were numerous, but did not form massive clumps; many bacilli showed altered staining reactions. No lesions were detected in organs other than the lung.

Sections were also examined from mice infected with 1 mg. or more of other avirulent strains. The rate of evolution of the lesions varied, but there was no evidence of any real difference in histological pattern or of the development of chronic, fibro-caseous lesions.

From the above findings it is obvious that a lowering of the inoculum or a fall in virulence of *M. tuberculosis* var. *hominis* is associated, in infected mice, with the substitution of proliferative for necrotic lesions, a foam-cell reaction in the alveoli, and longer survival; the infection is nevertheless progressive and unremitting in character.

Effect of vaccination.

Three groups of mice were inoculated intravenously with 0·0004, 0·002 and 0·01 mg. respectively of *M. tuberculosis* Strain 905. Thirty days later half the mice in each group were re-infected with 0·75 mg., the remainder being retained as controls. Mice from each of the 6 groups were killed at 70 days. Sections of lungs from the reinfected mice showed that the necrotic lesions characteristic of primary infection with 0·75 mg. were absent; in all groups the lesions were purely proliferative and, on histological evidence, the maximum protection was conferred by the smallest vaccination dose (Table III). The secondary infection was still, however, progressive and unremitting in its course.

TABLE III.—*Severity of Lesions in Mice Infected with* M. tuberculosis *Strain* 905. *Effect of Vaccination.*

Inoculum in mg.		Severity of lesions at (days).
Primary.	Reinfection.	
0·0004	—	4·3 (70)
0·0004	0·75	7·5 (70)
0·002	—	6·2 (70)
0·002	0·75	8·2 (70)
0·01	—	8·2 (70)
0·01	0·75	10 (70)
—	0·75	11 (21)

DISCUSSION.

The results show that the course of experimental tuberculosis and character of the lesions in mice are directly dependent upon the dose and virulence of the infecting strain of the bacillus. A large dose (0·75 mg.) of virulent bacilli causes death with necrotic lesions in about 25 days; smaller doses (0·02 mg. or less) permit longer survival, and cause proliferative rather than necrotic lesions. The pattern of evolution of the lesions conforms to that described and analysed in detail by Raleigh and Youmans (1948*b*).

It is possible, therefore, in terms of survival-time and duration of illness in infected groups of mice to define an " acute " disease produced by a large inoculum (0·75 mg.), and an apparently " chronic " disease produced by smaller inocula. Pathologically this difference is associated with necrotic lesions in the former and proliferative lesions in the latter type of infection. In both types, however, the disease is progressive, and there appears to be no final immunity or natural remission.

Large inocula (0·75 mg. or more) of various strains of *M. tuberculosis* of low virulence produce a disease comparable in every respect to that produced by smaller inocula (0·02 mg. or less) of the virulent Strain 905. Again, this apparently " chronic " disease differs from the " acute " only in its slower development, and in the prolongation of the proliferative stage of tissue-reaction. In both types, true caseation, fibrosis, cavitation and giant-cells are absent.

Vaccination with small inocula of living, virulent bacilli allows mice to resist reinfection for a longer period than unvaccinated controls. Such resistance is also associated with prolongation of the proliferative tissue-reaction, but it does not confer the power to arrest by fibrosis the development of the lesions or, in general terms, to cause remission of the disease. A small vaccinating dose is more effective than a larger dose. It seems likely, therefore, that the efficacy of vaccination in mice depends upon a balance between the resistance induced by the primary infection alone and the cumulative effect of the primary and secondary infections. It is of interest to note that, in mice, the primary infection is not even temporarily activated, and no allergic reactions are caused by reinfection. A similar observation was made by Pagel (1940), using mice infected intracutaneously.

In terms of the histogenesis of the disease, the resistance of mice to experimental tuberculosis is expressed firstly by an apparent decrease in the number of acid-fast bacilli in the lungs. This decrease is associated with interstitial infiltration by mono- and polymorphonuclear cells, within which bacilli with altered staining reactions may be observed. The infection is then quiescent for one or more weeks, depending upon the number and " virulence " of the bacilli in the original inoculum. Bacterial multiplication and proliferation of mononuclear cells then become apparent, and continue without remission until death occurs with necrosis or massive consolidation of pulmonary tissue. During the quiescent or early proliferative stages of the infection mice gain weight and look healthy. Loss of weight and decline in health are evident only when there is necrosis or massive consolidation in the lungs. Even with virulent infections no extensive lesions are observed in organs other than the lungs.

These general findings must be borne in mind when mice are used in the experimental chemotherapy of tuberculosis. Infection is always progressive, so that no false results need be expected from natural remission. With small inocula or avirulent organisms, however, there is some variation in the rate of progress of the disease, as instanced by the early deaths (necrotic lesions) and later deaths (proliferative lesions) in mice infected with 0·02 mg. Strain 905. Obviously, in a treated group, chemotherapeutic response to a non-toxic substance will be to some extent enhanced by the proportion of mice naturally destined to develop proliferative lesions.

Raleigh and Youmans (1948) noted a similar variation when mice were infected with 0·1 mg. human tubercle bacilli ; it is clearly of importance for each

investigator in chemotherapeutic work to establish the pathological course and define the variations of the experimental infection employed. In the experiments reported above, variation is minimal in the " acute " infection induced by 0·75 mg. Strain 905, and this infection should therefore provide an exacting test of therapeutic substances.

During the first 14 days of the " acute " infection morbid changes are minimal and bacilli are scanty and isolated. Intervention at this stage would presumably favour a drug rather than the bacillus. Treatment thereafter, when lesions are established, obviously decreases the likelihood of chemotherapeutic response and, with large inocula which overpower the resistance of the mouse, it may be impossible to intervene before a proportion of the mice have already developed lethal lesions. Delayed treatment may therefore be better demonstrated with " chronic " (e.g. 0·02 mg.) infections and, under these conditions, might reasonably simulate the treatment of established tuberculous lesions.

On the basis of their histopathological studies, Raleigh and Youmans (1948) formulated a system for assessing chemotherapeutic activity in experimental tuberculosis. Essentially, the " score " of each mouse is proportional to the amount of lung-tissue involved in the reaction to the infection, together with the number of bacteria and degree of involvement of liver, spleen and kidney. This system is undoubtedly carefully constructed and comprehensive, but in practice it is laborious, and it refers only to one specific infection (0·1 mg. *M. tuberculosis* Strain H. 37 RV).

The chief difficulty in devising or manipulating any such system is to know where to draw the line histologically between the immune and morbid components of the tissue reaction in infected lungs. According to Raleigh and Youmans the pathological unit should be " a more or less finite lesion, rather than general changes, such as congestion and oedema." Unfortunately, even a finite lesion may be purely proliferative in character ; and, in prolonged experimental infections (e.g. with small inocula, avirulent bacilli or as a result of chemotherapeutic suppression), the pathological changes may be diffuse and finite lesions absent.

Further difficulties arise over the changes in the spleen and kidney. Such lesions are usually proliferative with virulent strains and minimal or absent with avirulent strains. In the spleen the changes are completely diffuse, and it is by no means certain that the resulting splenomegaly is necessarily an indicator of the severity of the infection.

The alternative scheme of pathological assessment (Table I) used in our studies is based upon the following general principles :

(*a*) The number of bacteria present, and necrotic lesions in the lung, can both be regarded as absolute indicators of the advance of infection.

(*b*) Proliferative and exudative processes represent defensive reactions, and assume a morbid role only when 50 to 75 per cent of the lung tissue is replaced by them, whether as finite lesions or diffusely ; prior to this stage a limited score can be assigned to these processes to indicate the potential development of severe infection.

(*c*) Lesions occurring outside the lung are minimal or absent in " chronic " infections, and do not contribute significantly to the morbidity of the acute infection. Such lesions are, therefore, disregarded.

As in many other fields of experimental chemotherapy, it is important in anti-tuberculosis research to define the limitations as well as the assets of the

tests employed. Experimental tuberculosis in the mouse is a progressive disease, dissimilar in its course and morbid pattern to tuberculosis in man or cattle. It can, nevertheless, be readily standardized, and its progress at various stages can be easily assessed. With these reservations the experimental infection of mice with human strains of *M. tuberculosis* provides a reasonable basis for chemotherapeutic screening, though not necessarily for the final identification of compounds likely to be useful against tuberculosis in man.

SUMMARY.

Mice inoculated intravenously with virulent and avirulent human tubercle bacilli develop a progressive pulmonary infection.

0·75 mg. of virulent bacilli causes deaths with necrotic lesions in three weeks. Smaller doses of virulent bacilli (or large doses of less virulent bacilli) permit longer survival, and give proliferative rather than necrotic lesions.

Previous vaccination with living tubercle bacilli makes mice more resistant to reinfection. This resistance is expressed by the formation of proliferative lesions, and longer survival, but not by fibro-caseous lesions or remission of the disease.

In its pathological features, murine tuberculosis is dissimilar to human tuberculosis. Standardized murine infections may, nevertheless, lend themselves to "screening" tests in experimental chemotherapy. A scheme for pathological assessment of the experimental infection is presented.

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THE EFFECT OF ANIMAL PASSAGE ON THE PRODUCTION OF ANTIBIOTICS BY AEROBIC SPORE-FORMING ORGANISMS.

MARION C. SAVAGE AND H. W. FLOREY.

From the Sir William Dunn School of Pathology, Oxford.

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IT has been observed that the capacity of micro-organisms to produce antibacterial substances is often reduced by frequent subculture, a phenomenon causing much difficulty during investigation, and one which sometimes hampers the commercial production of antibiotics.

Weiland (1936) explored the idea that organisms which had lost their antibiotic-producing power might regain it by passage through the guinea-pig. He worked with strains, said to be *Bacillus mesentericus*, the colony appearance of which he described. He claimed to have obtained mucoid variants of high antibiotic-producing power by the animal passage of rough strains which previously produced only small amounts of antibiotic.

The experiments to be described were designed to ascertain whether Weiland's findings could be repeated when his procedures were followed as closely as possible.

EXPERIMENTAL.

Materials and Methods.

Culture media.

Heart broth, heart agar, blood agar, Loeffler's blood serum, potato dextrose broth such as were used for routine work in the laboratory, and Weiland's dilute lemco broth (Weiland, 1936), were used.

Organisms.

Since Weiland's strains were not available six *B. mesentericus* strains from the National Collection of Type Cultures (No. 1379, 1490, 1653, 2589, 5902 and 6223) were used, and two aerobic spore-forming rods which had been isolated from the air in Oxford (No. A3 and M103A). All the strains were uniformly rough before passage, and all appeared to be alike. They all produced acid, but no gas, in 3 days from glucose, sucrose and mannitol; there was no fermentation of lactose or maltose; gelatine and Loeffler's serum were liquified in 6 days; Voges-Proskauer and diastase tests were positive; indole was not produced in peptone water.

Strain A3 had produced moderate amounts of antibacterial substance four years earlier, but had almost entirely lost this ability during subculture. Strain M103A had a small amount of antibacterial activity.

Methods of detection and assay of antibiotic activity.

The streak method was used to ascertain the activity of the *B. mesentericus* strains. Preliminary tests showed no significant difference between their activity

against the pathogens that Weiland used and against *C. xerosis*. The latter strain was therefore used throughout as test organism. It was a subculture of a strain (No. 1042) obtained some months previously from the National Collection of Type Cultures.

Owing to natural variation the streak method proved unsatisfactory for comparing the antibiotic-producing power of organisms before and after passage, and the serial dilution method was found impracticable when numerous concurrent assays had to be made. Therefore, the horizontal diffusion method of assay known as the cylinder-plate technique was used for routine tests (Heatley, 1944).

Consistency of results in determinations of antibiotic activity.

Preliminary tests were done with Strain M103A, which produced some antibiotic before and after passage.

It became evident, from serial dilution tests, that there was considerable variation in the results obtained with different batches and types of medium, and between tests done on different days. This variation, if it chanced to occur with a strain which was in course of passage, might lead to the conclusion that passage was responsible for the change, unless all the tests were carried out concurrently and assay conditions were standardized. Therefore it was decided to grow all strains, both pre- and post-passage, simultaneously in duplicate flasks of Weiland's broth and potato dextrose broth, and to assay the medium by the cylinder-plate method against *C. xerosis* after two, five and eight days of incubation.

Passage Experiments.

The strains were passed through mice and guinea-pigs. A thick suspension of bacilli was obtained by suspending a culture on a blood agar plate in 2·0 ml. of heart broth. One third of a ml. of the suspension was injected into the tail vein of each mouse, and 0·5 ml. was injected intracardially into each guinea-pig. On killing the animals after 18 to 48 hours the spleen was removed with aseptic precautions, minced with scissors and forceps in a sterile petri dish, and cultured on blood agar.

TABLE I.—*Recovery of* Bacillus *from Spleen after Intravenous Injection in Mice.*

Experiment number.	Strain of *Bacillus*.	Hours after injection.	Colonies in culture from spleen. Number.	Types found.
	None (control)	..	1 col.	(Micrococcus).
1	M103A	24	+++	Rough.
			+	Mucoid.
2	,,	18	+	Rough.
			1 col.	Mucoid.
	,,	27	+	Rough only.
	,,	42	++	Rough.
			+	Smooth.
3	A3	18	+++	Rough.
	,,	27	+	Rough.
	,,	42	..	No growth.

+ = An average of less than 10 colonies per plate ; ++ = between 10 and 40 colonies per plate ; +++ = more than 40 colonies per plate.

TABLE II.—*Recovery of* Bacillus *from Spleen after Intracardial Injection in Guinea-pigs.*

Experiment number.	Strain of *Bacillus*.	Hours after injection.	Colonies in culture from spleen.	
			Number.	Types found.
4	A3	1 (died)	+++	Rough.
5	,,	24	+	Smooth.
			+	Rough.
6	M103A	48	+++	Rough.
7	N.C.T.C. 1653	48	+++	Rough.
	,, 2589	48	+	,,
	,, 1379	48	+	,,
	,, 1490	48	+	,,
			+	Smooth.
	,, 6223	48	+++	Rough.
	,, 5902	48	+++	Rough.

Signs as in Table I.

The colonies which grew are listed in Tables I and II. Regardless of varying colony form they were all identical with each other, and with the strains injected, by the biochemical tests that were used.

RESULTS.

The six N.C.T.C. strains produced no antibiotic activity before or after passage. In cultures from the spleen three of the N.C.T.C. strains gave smooth colonies as well as rough, but these were as inactive as their rough counterparts. The two laboratory strains showed much the same small yield of antibacterial substance after passage as they had done before.

SUMMARY AND CONCLUSIONS.

No change in the production of antibiotic resulted from the passage through animals of three different categories of *Bacillus*, i.e. strains that at no time produced any antibiotic, a strain that produced a slight amount, and a strain that had once produced an antibiotic but which had largely lost this capacity.

The only effect which could be discerned as a result of passage through an animal was the appearance, in a few cultures, of smooth and mucoid variants not seen in the original rough strains. These had no more antibacterial activity than their precursors. It is possible that the smooth strains were present in the original rough culture in very small numbers and became more prominent during passage, either because rough forms were killed more readily by the host, or for other reasons.

Thus it has not been possible to repeat Weiland's observations on the increased capacity of spore-forming organisms to produce antibiotics following animal passage.

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INDUCED BACTERIAL ANTAGONISM.

MARION C. SAVAGE AND H. W. FLOREY.

From the Sir William Dunn School of Pathology, Oxford.

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In a series of papers Schiller (1924, 1925, 1927, 1930), Schiller and Roublyova-Korabelnikova (1930) and Schiller and Adamovskaya (1931) reported experiments which they believed demonstrated that it was possible to induce one micro-organism to elaborate a substance or substances which were inimical to the growth of another when suitable cultural conditions were provided. For example, they grew yeast in a medium containing carbohydrate, in which the only source of nitrogen was the organism against which the development of an antibacterial substance was desired. Schiller claimed that in this way he produced substances which were capable of lysing the tubercle bacillus, and he suggested that by suitable manipulation of medium it should be possible to produce therapeutically valuable agents active against a number of pathogenic micro-organisms. This conclusion was contested by Wedenjapin (1925) and Barglowski (1938), who could not repeat Schiller's work and doubted whether bacterial antagonism could be induced.

A somewhat similar approach was made by Davide (1945), who grew *Proteus* and *Pseudomonas* in medium containing living tubercle bacilli, gradually reducing the content of peptone until the tubercle bacilli were the only source of nitrogen.

The method of " soil enrichment " was utilized by Dubos (1939) before he isolated *B. brevis*, the source of tyrothricin. He treated soil for 2 years by adding cultures of Gram-positive cocci, and at the end of that time isolated from it a strain of *B. brevis* that produced an antibiotic active against Gram-positive cocci. There was no evidence that the " soil enrichment " had played any part in the elaboration of the active strain. Waksman and Woodruff (1940) and Waksman and Schatz (1946) also used a " soil enrichment " technique, but without producing evidence for its efficacy, even though the organisms were subsequently transferred to agar plates incorporating living organisms, of the species used for the original soil enrichment. After the work recorded in this paper had been completed Risler (1948) reported that when *Aspergillus flavus* was grown in association with the tubercle bacillus it developed a specific enzyme which was capable of dissolving the tubercle bacillus. It was stated that this enzyme had protective effects against experimental tuberculosis of guinea-pigs. This work needs to be repeated, as the data given in Risler's publication were scanty.

From results so far published it is difficult to tell whether it is indeed possible to " induce " micro-organisms to produce antibacterial substances. The present paper describes an attempt to investigate the possibility further.

EXPERIMENTAL.

The experiments to be described were an attempt to test Schiller's hypothesis. Two bacterial species were placed together in a solution free from nitrogenous matter other than the cells of the organisms. According to Schiller, under such conditions one organism is induced to become an "antagonist," and to utilize for food the cells of the other, the "victim." Schiller asserted that strains of bacteria and yeast could be either antagonist or victim according to the conditions of culture.

Choice of Bacteria.

As none of Schiller's strains were available, an arbitrary choice of organisms was made on the basis of some theoretical considerations. If the antagonist is to be trained to utilize victim cells for food, then the shorter its mean generation time the sooner should results be obtained. Conceivably, proteolytic power might be advantageous to an antagonist by helping it to survive.

It was also of interest to see whether an existing antibiotic power could be enhanced by the same means.

With these considerations in mind, *Proteus vulgaris*, N.C.T.C. No. 5887, and two strains of *Bacillus licheniformis* were chosen. There was no evidence that the strain of *Proteus* produced any antibiotic substance, but the two strains of *Bacillus* had consistently produced small amounts for several months.

The choice of victim cells upon which the proposed antagonist was to feed was also arbitrary. If a new antibiotic was going to be produced as a result of these experiments, it would be useful to have one effective against some of those pathogenic bacteria most insensitive to known antibiotics. Infections caused in man by the tubercle bacillus and by Gram-negative bacilli have proved among the most refractory to the known therapeutic agents. On these grounds *Myco. tuberculosis* (H37Rv) was chosen as one victim and a common strain of *Bact. coli* (NCTC No. 86) to represent the Gram-negative organisms. A third victim representing a different bacteriological group was included, *C. xerosis*. The last was sensitive to the antibiotics produced by both of the *Bacillus* strains, and it was of some interest to compare the yield of antibiotics before and after contact with cells of a species known to be susceptible.

Methods of Bringing Antagonist and Victim into Contact.

Schiller planned his experiments on a short term basis, making his observations in a matter of a few hours or days. In his mixed cultures of bacteria in flasks one or other of the bacterial species soon died from lack of nutriment, or as a result of the accumulation of toxic metabolic products. On the other hand, if the cultures were maintained by transfer to fresh medium, the relative proportions of the two species were probably different at each transplantation.

To avoid the disadvantages of Schiller's method it was decided to present the potential "antagonist" to the "victim" in such a way that the latter could not overgrow the former. This was attempted in three different ways. First, the victim was killed and suspended in non-nutrient agar across the surface of which the antagonist was streaked ("bacterial agar" method). Secondly, in case living cells were required to induce antagonism, living tubercle bacilli were placed as victim with the *Proteus* antagonist in saline containing very small amounts of peptone. Under both these sets of conditions the victim could not

outgrow the antagonist, though in the second case it still remained alive. In these experiments the antagonist grew more slowly than usual, possibly because of a prolonged lag phase each time the organisms were transferred to fresh medium deficient in nutrients. In an attempt to produce a maximum number of generations in minimum time, a third method, by continuous culture in medium deficient in nutrients, was devised.

The three types of experiment, with bacterial agar, tubercle bacilli in dilute medium, and continuous culture apparatus, are described below.

EXPERIMENTS WITH BACTERIAL AGAR.

Preparation of Bacteria and Medium.

In these experiments *Bact. coli* and *C. xerosis* were used as victims, and *B. licheniformis* and *P. vulgaris* as antagonists. It was first necessary to investigate the minimum requirements for growth on agar medium. If the victim cells or the agar were insufficiently washed, the antagonist could live on the organic matter adhering to them without being forced to use the bacteria for food. On the other hand, if the victim cells and the agar were too well washed the antagonist might starve to death before any antagonistic power had been induced. Therefore, preliminary experiments were carried out to select a suitable compromise between these extremes. The following technique for preparing agar and cells resulted.

A hundred g. of agar were put in a muslin bag and washed for three periods of two hours in 10 gallons of tap water, with a final rinse in 5 l. of distilled water, after which it was dried at 37° C. The cells were obtained by thickly sowing one heart agar plate for every 8·0 ml. of bacterial suspension required. The plates were incubated at 37° C. overnight and the resulting growth rubbed up in 10 ml. of normal saline. The suspension was then centrifuged and the organisms washed twice with 10 ml. of saline. Normal saline was added to the packed washed cells until a 1/10 dilution had the same opacity as the third standard opacity tube of Brown's series (Brown and Kirwan, 1915). One ml. of this heavy suspension was added to 5·0 ml. of a 2 per cent solution of the washed agar in 0·85 per cent NaCl in tap water. The resulting suspension of cells in agar gave an opaque slope through which large print was just not decipherable. This enabled lysis to be estimated in terms of a gradual increase in the transparency of the slope. The pH of the medium was always between 7·0 and 7·4 before and after autoclaving at 120° C. for twenty minutes.

Detection of Antibacterial Activity.

At the beginning and end of each experiment all the antagonist cultures were streaked on heart agar plates (6 per cent agar for *Proteus*), and after incubation *C. xerosis*, *Staph. aureus*, *S. enteritidis* and *Bact. coli* were streaked at right angles to the antagonist. The antagonists were also subcultured directly from the bacterial agar into heart broth, potato dextrose broth, and Charrin and Dissard's (1893) basal salt solution enriched with asparagin and glucose. These fluid cultures were tested against *C. xerosis* and *Bact. coli* by the cylinder-plate method after two, five, and eight days' incubation at 37° C.

Experiments Performed.

The antagonist strains, *Bacillus* and *Proteus*, were grown in duplicate on bacterial agar slopes under the different conditions listed below :

1. Three subcultures at 48-hour intervals, incubated at 37° C.
2. Twenty subcultures at weekly intervals, incubated at 37° C.
3. Five months' incubation at 37° C.
4. Three days' incubation at 37° C., then five months' storage at 4° C.
5. Three days' incubation at 37° C., then five months' storage at room temperature.
6. Five months' incubation at 24° C.
7. Five months' storage at room temperature.
8. Five months' incubation at 37° C., followed by 20 subcultures at weekly intervals.

All these tests were controlled by simultaneous culture of the organisms on heart agar and saline agar without cells.

Three criteria were used to detect possible change in the antagonists as a result of these various treatments. Firstly, their ability to utilize as food the protoplasm of the victim cells, as indicated by their rate of growth ; secondly, their ability to lyse the victim cells, as estimated by clearing of the bacterial agar ; and thirdly, their ability to produce antibiotic activity against the victim or against other bacteria.

Results.

Both the antagonists produced abundant growth on bacterial agar in less time at the end of each experiment than at the beginning, showing that some form of adaptation had occurred, possibly involving utilization of the bacterial cells.

Their ability to lyse the victims' cells varied. *Proteus* was consistently unable to lyse either *Bact. coli* or *C. xerosis*. The *Bacillus* lysed *Bact. coli* more rapidly at the end of the experiments than at the beginning, but no antibiotic activity against the living organism could be detected in liquid cultures. *C. xerosis* cells were lysed very slowly by the *Bacillus* at first, but prolonged storage followed by repeated subculture increased the rate of lysis, and did so more quickly than did storage or subculture alone.

It will be remembered that the *Bacillus* strains originally produced small amounts of antibiotic against *C. xerosis*. These small yields were not increased as a result of any of the experiments, nor was there any significant difference between the antibiotic yield of these organisms grown on bacterial agar, and of the same strains grown on heart extract or saline agar.

Conclusion.

As the result of growth on bacterial agar under the conditions of experiment the antagonists chosen appear to acquire some degree of increased ability to use the substance of the bacterial victims, as shown by improved rate of growth and, in the case of the *Bacillus*, by increased capacity to lyse the victim cells. However, this acquired ability does not appear to be associated with any increased ability to produce antibiotic substances. The potential antagonist chosen which did not possess this capacity at the inception of the experiments had not acquired it at their completion. The antagonist which initially showed a small amount

of anti-*xerosis* activity gave no evidence of any increase in the amount of antibiotic as a result of the experiments, though under some conditions greater facility in lysing *C. xerosis* was acquired; consequently the production of this antibiotic appeared to be unrelated to the ability of the antagonist to lyse bacterial cells.

EXPERIMENTS WITH TUBERCLE BACILLI.

These experiments were designed to repeat part of Davide's work (1945, 1946), using as far as possible the methods which he described. The *Proteus* and *Pseudomonas* were grown by him in peptone broth containing living tubercle bacilli, and periodically transferred, mixed with the tubercle bacilli, to fresh medium, the concentration of peptone being reduced at each transfer until the cells of the tubercle bacillus were the only source of carbon and nitrogen. Because Davide found slight inhibition of the tubercle bacilli by "unadapted" *Pseudomonas*, *Proteus* was chosen for the present work to make error less likely.

Preparation of Bacteria and Medium.

Davide's paper gave few details of the methods used, so preliminary experiments were made to find out how his principles could best be applied.

The first problem was to secure living tubercle bacilli growing discretely enough to be properly washed and easily available to the antagonist. Dubos and Davis's (1946) modification of Kirschner's medium yielded a large number of acid-fast organisms in finely particulate form in a short time, and, given roughly the same inoculum, the weight of organisms obtained in 8 days was about the same in each bottle of a batch of medium. The weight per bottle was at least 50 mg. in each of the six preliminary tests done. Before use the bacilli were washed six times with 10 ml. volumes of distilled water, being centrifuged between each washing for twenty minutes with a centrifugal force calculated to be about 3000 times gravity. This was facilitated by growing the organisms in screw-capped 1-oz. bottles which could be placed directly in the centrifuge cups.

The initial concentration of Evans' peptone in the medium to be used was the smallest amount upon which *Proteus* would develop a faint trace of turbidity in 48 hours at 37° C. During the course of the experiment the amount was gradually reduced, the smallest concentration reached being 0·0002 per cent.

The *Proteus* for inoculation was prepared in the same way as cells for incorporation in bacterial agar, except that the final suspension for inoculation was standardized to match Brown's standard tube No. 1.

Detection of Antibacterial Activity.

To ascertain whether any antibiotic was being developed as the experiments proceeded a rapid assay method was required. There was in the laboratory a strain of *Myco. phlei* which would produce confluent growth overnight on heart agar. This organism, with a variety of other test organisms, was used for rapid assay by the streak test, on the chance that any antibiotic produced against tubercle bacilli would inhibit this species of *Mycobacterium* as well, as did Callow and Hart's licheniformin (1946).

In addition to this rough test on solid medium an accurate assay method for activity against mycobacteria was required. For this, the antagonist which had been in contact with the tubercle bacillus was subcultured into duplicate

flasks of heart broth and Charrin and Dissard's basal salt solution. After two and five days' growth the contents of the flasks were sampled and assayed by the cylinder-plate method against *Myco. phlei*. After seven days' growth the material in the flasks was sterilized by passing through sintered glass filters. The filtrates were added in 0·5 ml. amounts to duplicate bottles containing 9·5 ml. of Dubos medium. Approximately 0·5 mg. of living tubercle bacilli (from cultures in Dubos medium) was added to each bottle, and the bottles were then incubated at 37° C. for two weeks.

Experiments Performed.

Each week four cultures containing approximately 50 mg. of tubercle bacilli each were washed by the method described. One bottle of washed organisms was kept as a control, and to it were added 4·9 ml. of saline without peptone and 0·1 ml. of the suspension of washed *Proteus*. To each of the other three bottles were added 0·3 ml. of a 0·1 per cent peptone solution (final concentration of peptone 0·006 per cent), 4·6 ml. of saline and 0·1 ml. of *Proteus* suspension. The 0·006 per cent peptone solution was also set up with *Proteus* only, and another control was set up with *Proteus* in saline.

Each week, after shaking the bottles, 0·1 ml. of the bacterial suspension was transferred from each to a bottle of fresh dilute medium having a similar constitution except that the concentration of peptone was reduced by stages. As a precaution against *Proteus* dying out in the diluted medium 1 ml. of bacterial suspension instead of 0·1 ml. was transferred each week in the two control series containing *Proteus* without tubercle bacilli.

The bottles were examined visually at two, four and seven days, by means of the usual bar-light device, to ascertain whether any growth of *Proteus* had occurred. Immediately after inoculation there was no turbidity, the tubercle bacilli being clumped as a sediment in those bottles that contained them.

In the beginning the concentration of peptone was reduced by approximately 25 per cent each week. When it had fallen to 0·002 per cent the control containing *Proteus* alone with peptone was clear for the first time. This suggested that it might be wise to reduce the peptone less often, so a 50 per cent reduction was made fortnightly.

Five days after each transfer a sample from each bottle was plated out to test the viability, purity and cultural characteristics of the *Proteus* before the next transfer.

Results.

Table I shows the amount of growth made by *Proteus* 4 days after each of the 14 transfers carried out. Three times the period between transfers had to be extended to 10 days, owing to contamination. When this happened the *Proteus* was purified by plating on heart agar. Its ability to grow under the artificial conditions of the experiment was not affected by such a passage.

It is clear from Table I that in the later part of the experiment *Proteus* was making better growth on dilute peptone with tubercle bacilli than on dilute peptone alone. Thus it is possible that it had to some extent become adapted to utilize the tubercle bacilli. It seems likely that the presence of peptone is not essential for such adaptation, as Davide thought, since in Periods 12 and 13 there was definite slight turbidity due to the growth of *Proteus* in control bottles containing saline and tubercle bacilli only.

TABLE I.—*Growth of* Proteus *in Diluted Medium or Saline, With or Without* Myco. tuberculosis, *as Shown by Turbidity of Supernatant Fluid after* 4 *Days' Incubation.*

Period number.	Concentration of peptone in diluted medium. Per cent.	In diluted medium. With *Myco. tuberculosis* (3 replicates). 1.	2.	3.	Alone.	In saline. With *Myco. tuberculosis*.	Alone.
1	0·006	++	++	++	+	Clear	Clear
2	0·005	++	++	++	+	,,	,,
3	0·004	++	++	++	+	,,	,,
4	0·003	+	++	++	+	,,	,,
5	0·002	+	++	++	+	,,	,,
6	0·002	+	+	+	Clear	,,	,,
7	0·001	++	++	++	,,	+	,,
8	0·001	+	+	+	,,	Clear*	,,
9	0·0005	+	+	+	,,	Clear	,, *
10	0·0005	++	++	++	+	,,	,,
11	0·0002	+?	+	++	Clear	+?	,,
12	0·0002	+	++	+	,, †	+	,,
13	None	+	+	++	..	+	,,
14	,,	+?	+?	+?	..	+?	,, *
15	,,	+?	+?	+?	..	+?	,,
						Clear*	

\+ Very faint turbidity; ++ faint turbidity.
* *Proteus* dead, replaced by 1·0 ml. from bottle of previous period.
† *Proteus* dead, series discontinued.

Interesting colony variants of *Proteus* appeared in the routine cultures made for checking viability and sterility before each transfer. The swarming character of the *Proteus* became less pronounced with each transfer, and thicknesses in the spreading film due to individual colonies developed more frequently. After two transfers, two variants with discrete colonies could be observed:

(1) Colonies 1 mm. in diameter at 18 hours on heart agar, smooth surface, entire margin, convex, opalescent; organisms sluggishly motile in heart broth after 8 hours at 37° C.

(2) Similar to (1) but colonies 0·5 mm. in diameter; organisms non-motile in heart broth at 12 hours. Subcultures from the heart broth produced larger colonies composed of motile organisms, which later showed a tendency to spread.

These two variants appeared both in the presence of tubercle bacilli and in the controls without tubercle bacilli. When the cultures were purified on account of contamination at Periods 6 and 10 spreading *Proteus* only was re-introduced. By Periods 8 and 12 the variants had reappeared.

These variants were similar to those which Vanni (1933) described in a X19 *Proteus* strain growing in glycerine broth with and without tubercle bacilli. His non-motile small colony variant appears to have remained non-motile, however, upon subculture.

All the variants from each bottle were tested by the streak method for anti-

biotic activity at the end of Periods 4, 8 and 12, but none inhibited any of the test organisms used.

The ability of the antagonist to produce an antibiotic against mycobacteria was tested after Period 12. None of the colony types showed any inhibition of *Myco. phlei*, and in every instance sterile filtrates of the cultures failed to inhibit the growth of tubercle bacilli in Dubos medium.

Conclusion.

Although *Proteus* was apparently trained to utilize tubercle bacilli as a nutrient, there was no evidence that, under the conditions used, the acquisition of this power was accompanied by formation of an antibiotic. It must be recognized, however, that the detection of antibiotic activity against the tubercle bacillus is a difficult matter, and that the methods used were arbitrary and partly indirect. Davide's belief that in order to become adapted to utilize tubercle bacilli *Proteus* must be provided with peptone at first is not altogether confirmed.

The morphological variants of *Proteus* which appeared during the tests appeared also in controls without tubercle bacilli. This is similar to Vanni's findings.

EXPERIMENTS USING CONTINUOUS CULTURE APPARATUS.

The object of these experiments was to assist the production of an antibiotic by maintaining the victim and antagonist together under conditions in which the antagonist was kept continuously in the logarithmic phase of growth. In the apparatus described, a drop of fresh " victim cell medium " was added at frequent intervals to a larger drop of the same medium inoculated with the antagonist. The volume of the drop was kept roughly constant by the periodic removal of fluid. If the acquisition of antibiotic-producing power depends on the occurrence of the right mutation, the chances of this happening are greater if, as is assumed, the constant renewal of medium enables more generations to develop in a given time than under more usual conditions of culture.

Continuous Flow Apparatus.

The apparatus used was based on that described by Levin (1944), in which a *Penicillium* was grown on a loop composed of several coils of fine wire, and the loop was fed with fresh sterile medium by drops from a wick siphon. The wick feed was unsuitable for the present work, partly because the bacterial cells in the incoming medium might clog the flow, and partly because the rate of flow could not be made small enough to avoid washing away the antagonist bacterium. The wick was therefore replaced by a syringe and needle. Sims and Jordan (1942) used an automatic syringe to feed a continuous culture apparatus, but its force and rapid rate of operation rendered it useless for this work. Similarly Noble's syringe mechanism (1946) proved unsuitable. A device which pushed the syringe plunger at the required speed was specially designed and constructed by Mr. S. W. Bush.

Continuous flow system.

The apparatus, which is shown in side view in Fig. 1, comprises the following parts : An electric clock motor (A) of the synchronous type is connected by a

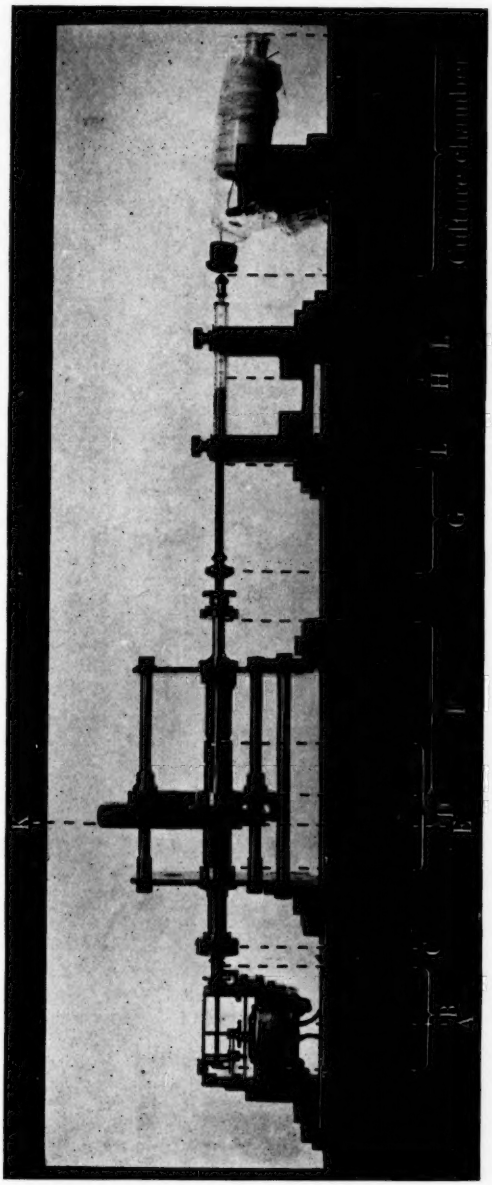

FIG. 1.—Continuous flow apparatus and culture chamber (approx. ½ of actual size). For description see text. Details of the culture chamber are shown in Fig. 2.

train of gears (B) and a safety clutch (C) to a threaded rod (D). A mit (E), travelling on the latter, imparts a slow horizontal thrust through a rod (F) to the plunger (G) of the syringe (H). The mit (E) is split, the two halves being pressed together by a spring, and by separating the halves (by pressing the lever (K)) a quick return mechanism is obtained. The threaded rod (D) has twenty-six threads to the inch and is driven at the rate of one revolution in thirty minutes. Almost 27 hours were required to drive rod (F) through its stroke and expel the whole contents (1·1 ml.) of a tuberculin syringe. For filling and cleaning the syringe is readily removed from the blocks (L), in which it is rigidly clamped.

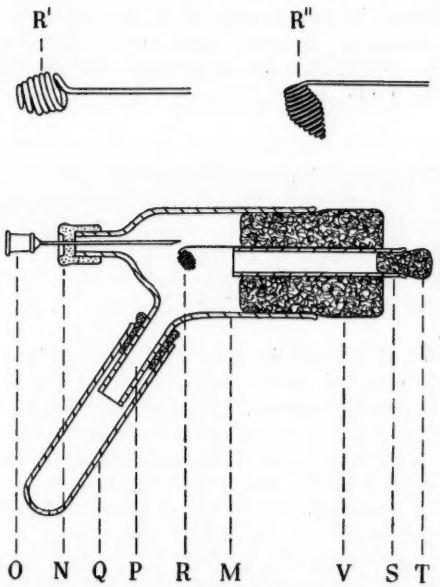

FIG. 2.—Culture chamber of continuous flow system. R′ and R″ show detail of loops. For description see text. (Chamber approx. $\frac{2}{3}$ of actual size; loops $\frac{3}{5}$ of actual size.)

Culture chamber.

The culture chamber, which is shown diagrammatically in Fig. 2, was constructed from a Pyrex test tube (M). The closed end was drawn out into a narrow tube and the tip cut off. This end was covered with a rubber vaccine bottle cap (N) turned inside out, which provided a sterile stopper through which the syringe needle (O) could be inserted. At the side near this end of the tube a small piece of glass tubing (P) bent sideways was inserted. The end of this was covered with a dwarf test tube (Q) sealed off by cotton wool around the tubing. The dwarf tube served to collect the waste products as they fell from the loop (R).

The straight end of the wire bearing the loop (R) was tied firmly with cotton thread on to a short piece of wide glass tubing (S). This tubing with the wire attached was plugged with cotton wool (T) at the outer end, and encased in cotton

wool and gauze (V) secured with cotton thread. The whole formed a firm plug with the wire and its loop protruding in one direction and a short piece of plugged tubing in the other. This plug was placed in the open end of the original test-tube so that the loop came directly underneath the end of the needle. Thus the fluid expelled from the syringe fell on to the loop, and the overflow was collected in the dwarf tube. Before use the assembled chamber was autoclaved at 120° C for 20 minutes.

Syringe.

One ml. Accoson all-glass tuberculin syringes were coated inside with liquid paraffin before sterilizing in the hot air oven, in order to reduce friction and leakage around the plunger. A needle with 0·813 mm. external diameter was used (Asco No. 245). From this the apparatus delivered, in 38 minutes, one drop of approximately 0·025 ml.

Loop.

It was felt that the antagonist would be less likely to be washed away from a large loop than from a small one. Therefore, efforts were directed towards producing the largest loop in which an aqueous suspension of bacteria would remain. Such a loop was wound by machine in Nichrome wire 1·219 mm. thick and held 0·3 ml. of water (R′). Theoretically, when the machine was running little of the original medium would remain in the full loop after 12 drops had fallen, which took about 8 hours.

The actual rate at which bacteria were washed out of the loop was measured by plunging it into 3·0 ml. of distilled water and estimating the opacity of the water in a Spekker absorptiometer. Twenty-four hours' running time with water in the syringe was required before the diluted loop fluid ceased to have detectable opacity. The persistence of bacteria for so long a time was probably due to some being trapped in the interstices between the coils, and was an asset in that it increased the possibility of the antagonist surviving until it had learned to utilize living victim cells. When it had been found that the loop retained cells for so long a smaller loop was devised (R″). This was made of Nichrome wire 0·508 mm. thick and held approximately one half of the volume of the original loop. It had double the number of coils, and so could retain bacteria in the interstices even though the medium passed through it twice as quickly as through the original loop.

Preparation of Bacterial Suspensions.

The strain of *Bact. coli* used in the previous experiments was chosen as victim and the strain of *P. vulgaris* as antagonist. On the basis of convenience and a maximum viable count the *Bact. coli* was grown at 24° C. for 16 hours on heart agar. The cells were then washed in the manner described above and suspended at a density of 1000 million cells per ml. in 0·027 M phosphate buffer, pH 7·2, containing 0·5 per cent "Analar" glucose. The syringe was filled with this, put in place, and refilled every 24 hours.

The *Proteus* was grown and washed in the same way as the *Bact. coli*, and suspended in distilled water, and the approximate number of cells was estimated by comparison with Brown's tubes. Densities ranging from 400 to 9000 million

cells per ml. were used in different experiments. The loop was filled with the suspension by means of a bent Pasteur pipette introduced through the glass tubing (s). Each day the contents of the loop were sampled by pipette and cultured on heart agar.

Detection of Antibacterial Activity.

At the end of each experiment, and at 7-day intervals in the longer ones, *Proteus* from the loop was tested for antibacterial activity. Cultures in heart and potato dextrose broth were tested against *Bact. coli* after 2, 5 and 8 days' incubation by the cylinder-plate technique. Streaks on 6 per cent agar plates were cross streaked with the test organisms used previously.

Experiments Performed and Results.

A preliminary experiment was first carried out with the large loop to ascertain how long *P. vulgaris* at an initial concentration of 6000 million per ml. would survive when fed with sterile glucose buffer solution. The plate culture showed typical spreading *Proteus* after 48 hours. At 3 days a thicker non-spreading variant with a colony 1·0 mm. in size appeared. After 5 days there was only a tiny 0·5 mm. non-spreading colony. On the 7th day the culture was sterile. The variants were similar to those which appeared in the experiments with tubercle bacilli, and those which Vanni (1933) described.

It was surprising that the bacteria should have persisted in the loop for as long as 6 days when the fluid was constantly being changed, but the persistence was probably due to some bacteria remaining in the interstices of the coils. Since there was no way of estimating the numbers so placed, it was apparent that counts made while an experiment was running would not give an accurate picture of the numbers of organisms on the loop. Quantitative estimates were not attempted, therefore, and attention was focused on the qualitative differences which might be observed as a result of continuous culture.

The variants described above were seen many times in subsequent cultures, and one observation was continually repeated. Most of the cells of the *Proteus* cultures introduced into the loop were actively motile, but without exception they lost this property after 24 hours.

More than 20 experiments were carried out in all. Several were cut short by the loop draining spontaneously owing to slight maladjustment, and contamination was difficult to avoid when the syringe was replaced and the loop sampled each day.

With the larger loop, and *Bact. coli* washed only twice, so that traces of peptone may have been present, *Proteus* was present for 16 days in one experiment and 23 days in another. With the smaller loop, through which the *Bact. coli* suspension passed more quickly, and with *Bact. coli* washed three times, the *Proteus* survived for 9 days in one instance and 11 days in another.

It will be seen that technical difficulties were considerable, and the number of successful runs that were finally accomplished was not great. However, in these four experiments the two organisms were successfully kept alive and in contact for periods of from 9 to 23 days. No antibiotic production by either the spreading *Proteus* or its variants could be detected at any time.

Conclusion.

Proteus vulgaris can survive for at least 11 days in a continuous culture apparatus fed only with *Bact. coli* cells (living and dead) suspended in phosphate buffer containing glucose.

No change in the antagonistic properties of *P. vulgaris* against *Bact. coli*, or any of the other test organisms used, could be observed as a result of continuous culture in the presence of *Bact. coli*.

DISCUSSION.

Although it is common to find that micro-organisms become resistant to antibiotics when grown in their presence, there is little convincing evidence that one micro-organism may be induced to form an antibiotic acting against another when the two organisms are grown together. It may be thought that the latter type of microbial variation is inherently less likely than the former, and it is certainly true that one mechanism by which a culture might become resistant to an antibiotic (natural selection) would not be sufficient for the formation of a culture producing an antibiotic. Nevertheless, the possibility of induced antibiotic production cannot be dismissed *a priori*.

A micro-organism might conceivably acquire the ability to produce an antibiotic, as it might acquire resistance to an antibiotic, in two ways : (1) by chance mutation affecting a very small proportion of the cells in a culture, or (2) by a change in the majority of the cells stimulated by substances in their environment.

(1) A mutation might occur spontaneously, or it might in theory be induced by a mutagenic agent from the victim. Mutations yielding a few cells resistant to an antibiotic are automatically followed by the selection of a resistant strain, since the growth of the sensitive organisms is suppressed by the antimicrobial agent. But if a mutant is formed which produces an antibiotic, its growth will not necessarily be favoured in comparison with that of the inactive strain, and in the absence of selection the mutation will not result in the emergence of an active culture. For selection to come into play it would be necessary for the mutant not only to produce an antibiotic, but to grow preferentially on metabolic products of the victim or on degradation products of the victim resulting from the action of the antibiotic. The chance conjunction of these circumstances may seem rather improbable.

(2) If a change occurs which leads to the production of an antibiotic by the majority of the cells in a culture selection will clearly be unnecessary.

It is possible to imagine a case in which specific substances diffusing from the victim are essential components of a medium in which the antagonist can produce an antibiotic. The role of the victim would then be to provide the *recherché* medium required for antibiotic formation. This unlikely occurrence could hardly be regarded, however, as an example of induced antibiotic production in the sense intended in the present paper, since it would not be due to an inheritable change in the antagonist.

On the other hand, substrates liberated from the victim might well stimulate the formation of extracellular adaptive enzymes by the antagonist. Adaptive enzymes from micro-organisms are relatively common. If the substrate were a macromolecule forming an essential part of the cell surface of the victim, or a specific intermediate necessary for its growth, the adaptive enzyme could act as an antibiotic.

It must be remembered, however, that the ability of an organism to produce an adaptive enzyme may be lost when it is grown in the absence of the substrate, and also that enzymes known to kill or prevent the growth of micro-organisms are rare. Enzymes which lyse bacteria that have been damaged by other agents often have no discernible effect on the intact cell. An adaptive enzyme discovered by Dubos, which dissolves the capsule of the pneumococcus, destroys the virulence of this organism, but not its ability to grow. Apart from the non-specific notatin, no spontaneously produced antibiotic has yet been proved to have enzymatic properties. Since adaptive enzymes are probably not formed *de novo* but are merely synthesized in much larger amounts in the presence of a substrate, this does not encourage the belief that adaptive antimicrobial enzymes will frequently be encountered.

The results of the present work do not disprove the possibility that one micro-organism may induce the formation of an antibiotic by another, but they do throw doubt on claims that such a phenomenon can easily be demonstrated.

GENERAL CONCLUSION.

Three different methods have been used in the attempt to induce some bacteria to form substances antagonistic to other species. There has been no success.

An apparatus for the continuous culture of bacteria, by which they are maintained in the log phase, is described.

We are much indebted to our colleagues, Dr. M. A. Jennings, Dr. E. P. Abraham and Dr. G. P. Gladstone, for help in the arrangement of this paper and in the formulation of the discussion of the results. We thank Miss Mavis Bond for technical assistance.

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SURFACE AND SUBMERGED GROWTH OF *COPRINUS QUADRIFIDUS.*

DOROTHY GILL-CAREY.

From the Sir William Dunn School of Pathology, Oxford.

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WILKINS and Harris (1944), Wilkins (1945, 1946, 1947*a*, 1947*b*, 1947*c*, 1948), Robbins, Hervey, Davidson, Ma and Robbins (1945) Atkinson (1946), Mathieson (1946), Melin, Wikén and Öblom (1947), Wikén (1947), Wikén and Öblom (1947), and Hervey (1947) have tested many hundred species of Basidiomycetes for the production of antibiotics, and have reported that about 40 per cent show antibacterial activity. In these investigations sporophore extracts, cultures on agar or culture fluids obtained by growing the fungi on liquid medium in small flasks were used. Before the antibiotics can be further investigated it is necessary to produce sufficient raw material from which they can be extracted by chemical manipulation. Lactaroviolin (Willstaedt, 1935) and polystictin (Chain and Callow, 1943, unpublished) were obtained from sporophores, but the easiest way is to cultivate the basidiomycetes on liquid medium. This has been done on a scale sufficient for chemical purposes by Falck (1923), Ehrenberg, Hedström, Lofgren and Takman (1946), Hollande (1947), Rivière, Thely and Gautron (1947), Robbins, Kavanagh and Hervey (1947), Bendz, Wallmark and Öblom (1948) and Kavanagh, Hervey and Robbins (1949).

The methods which have so far been described depend upon obtaining a growth of the basidiomycete on the surface of a shallow layer of medium contained in some form of large flask. The growth of most basidiomycetes is slow in culture, but the production of active fluids has been speeded up as much as about 4 times by using already formed mats of mycelium. Robbins *et al.* (1947) and Kavanagh *et al.* (1949) grew the fungus on beech shavings with one litre of medium in conical flasks. The original culture fluid was drained from underneath the mats, and replaced by fresh medium. Mats were reflooded many times, but were not usually kept for longer than 6 months.

The following paper describes methods which have been used for growing *Coprinus quadrifidus* both by surface culture and by submerged culture—a procedure so far not apparently used for the production of antibiotics from basidiomycetes.

EXPERIMENTAL.

Source of culture.

The culture was obtained from Dr. W. H. Wilkins, of the Department of Botany, Oxford University, who reported that when the fungus was grown on a 5 per cent malt extract the fluid showed activity against *Staph. aureus* and *Bact. coli* (tested by the hole plate method).

Stock cultures.

These were grown on 5 per cent malt extract agar slopes at 24° C. and stored at 4° C.

Methods of assay.

A preliminary small scale experiment in 50 ml. Erlenmeyer flasks indicated that the activity of the culture fluid could be determined by the cylinder plate assay method, using plates surface-seeded with a standard dilute suspension of *Staph. aureus* (N.C.T.C. No. 6571) (Heatley, 1944).

Surface Culture.

This was carried out in glass vessels designed by Glaxo Ltd.

Methods of inoculation.

(a) *From solid medium.*—The inoculum was prepared from plate cultures grown on 5 per cent malt extract agar and incubated at 24° C. It was found that if the agar medium was less than 3 mm. deep, or if the cultures were incubated for longer than about three weeks, the agar became leathery or horny and difficult to divide. After the mycelium had covered the plates (about 15 days) the cultures were stored at 4° C. until needed. No loss in viability was demonstrated in cultures stored for several weeks. For the first experiments the whole culture was divided into discs 1·5 cm. in diameter by means of a sterile cork borer, and these were transferred to the surface of the medium in the vessels on a long sterile needle This method was found uneconomical for large scale experiments, but was useful when uniform or graded amounts of inoculum were required. In later work the mycelium was cut roughly into squares by means of a sterile scalpel.

Both the discs and the squares showed a tendency to sink to the bottom of the medium within a few hours of inoculation, and although a little growth occurred in this position, it was very slow. Development of surface mats and production of antibacterial activity was greatly retarded if not altogether absent. This appears to be a fairly general property of basidiomycete cultures, and the difficulty can be met by supporting the inoculum in some way on the surface of the medium. The addition of 0·2 per cent agar to the liquid medium or a pad of non-absorbent cotton wool was found satisfactory. With *Coprinus quadrifidus*, the difficulty was overcome by incorporating granulated cork in the agar of the plate culture. The most satisfactory results were obtained with 16 mesh cork, between 5 and 10 ml. of which were incorporated in the agar of each plate. The cork was dry-sterilized inside an empty petri dish, and was mixed thoroughly with the agar (20 ml.) while this was being poured. The mycelium grew well on such plates, and the inoculum prepared from them always floated on the liquid cultures. In a further development of this method the culture was cut with a sterile scalpel into fragments about 2 mm. square, which were transferred to to the vessels by means of a long narrow metal shovel. The fragments spread over the surface of the medium, and as they all floated there were many centres of growth so that the surface was rapidly covered.

(b) *From liquid medium.*—In this method the inoculum was prepared from mycelial mats already established in vessels. The culture fluid was poured off

aseptically, and the mat was torn into fragments by means of a long sterile hooked wire and transferred to a new vessel. Such fragments always floated, the growth from them was rapid and the surface of the medium was quickly covered.

The procedure finally adopted was to inoculate most of the vessels of each batch in this manner, but to start 10 to 15 vessels from agar plates. The mats derived from these vessels were, when mature, used to inoculate the bulk of the next batch.

Size of inoculum.

The length of time taken for activity to appear in the medium was reduced when the surface was covered rapidly, so that a large inoculum was advantageous.

Depth of medium.

A depth of 2 cm. was used. With deeper layers the production of active material was considerably delayed.

Composition of the medium.

Cultures were grown on malt extract medium containing 1·25 per cent, 2·5 per cent, 5 per cent, 7·5 per cent and 10 per cent of malt extract. Growth and antibiotic production were best in 2·5–5 per cent malt extract, and were retarded to varying degrees in the more concentrated media. Other media such as those containing corn steep liquor, yeast extract, peptone, and potato were less satisfactory.

Temperature.

In a series of vessels incubated at 17° C., 24° C., 30° C. and 37° C. the greatest production of activity occurred in those incubated at 24° C. and 30° C. At 30° C. the initial rate of production of antibiotic was greater than at 24° C., but after a few days' incubation cultures at 24° C. had overtaken those at 30° C. Since it was more convenient to work at 24° C. this temperature was used.

Reflooding.

Vessels containing layers of 5 per cent malt extract medium 1 cm. deep appeared to reach maximum activity in approximately 12 days. At this point the

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EXPLANATION OF PLATES.

FIG. 1.—To show the effect of different amounts of aeration on growth and activity of *Coprinus quadrifidus* in submerged culture.

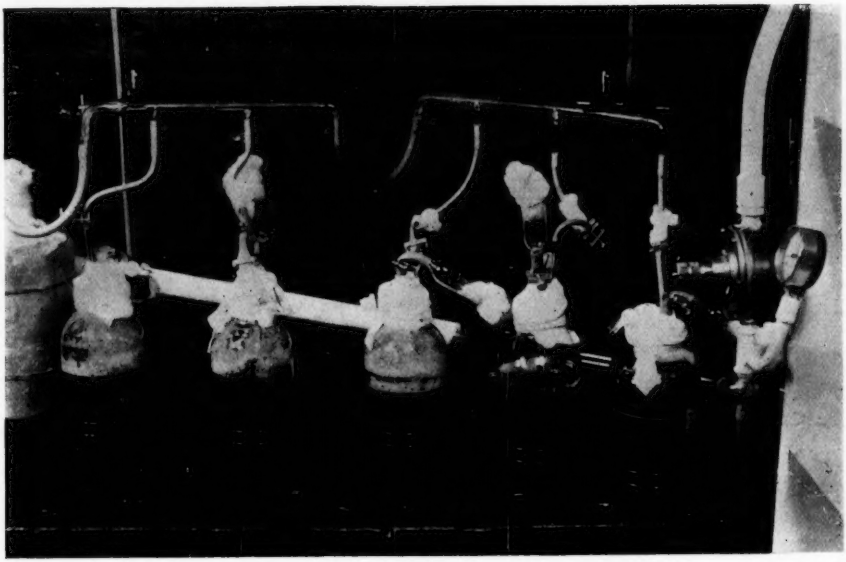
FIG. 2.—Appearance of growth after 9 days' incubation. Three litres of air per litre of medium per minute. Growth of the mycelium in the form of small " balls."

FIG. 3.—Appearance of growth after 9 days' incubation. Two litres of air per litre of medium per minute. " Balls " slightly larger.

FIG. 4.—Appearance of growth after 9 days' incubation. One litre of air per litre of medium per minute. " Balls " similar in size to those in Fig. 3.

FIG. 5.—Appearance of growth after 9 days' incubation. Half a litre of air per litre of medium per minute. " Balls " larger.

FIG. 6.—Appearance of growth after 9 days' incubation. A trace of air per litre of medium per minute. Scant growth. Some mycelium was demonstrated on the bottom of the bottle and some on the bubbler.



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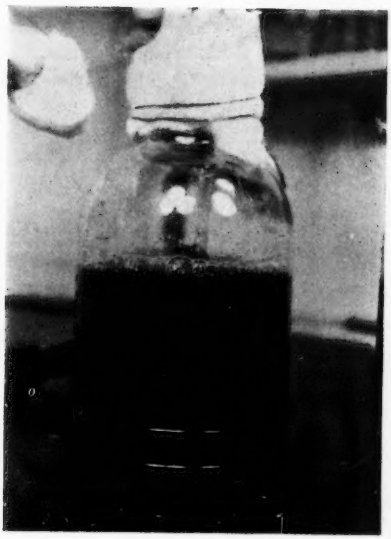

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culture fluid was poured off aseptically and new medium was introduced beneath the mat. The same order of activity as was found in the first batch of medium was reached after reflooding in 6 days. Reflooding was successfully repeated six times before the experiment was terminated.

Submerged Aerated Cultures.

Growth in submerged aerated cultures.

Apparatus (Fig. 2 and 3).—Submerged cultures of *Coprinus quadrifidus* were grown in 1-litre bottles and in 10-litre bottles. Both sizes of bottle were fitted

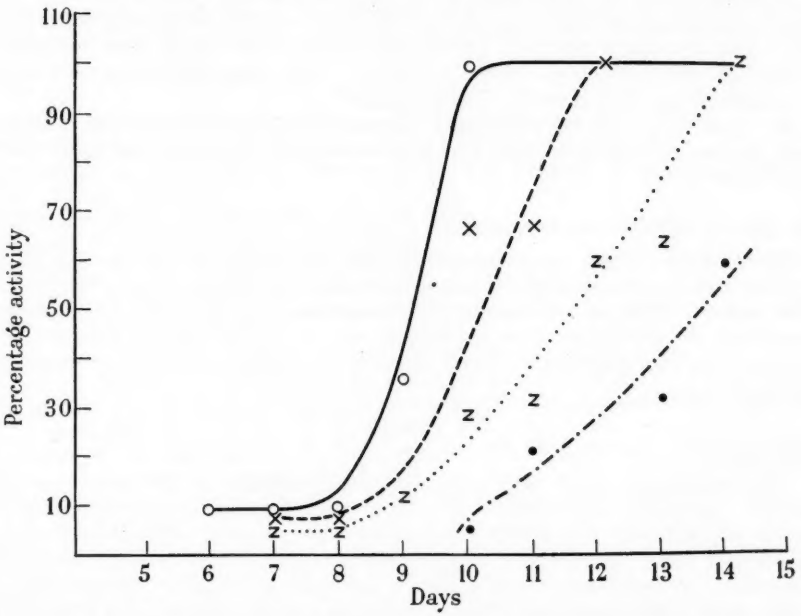

FIG. 7.—The course of production of antibiotic activity by submerged cultures of *Coprinus quadrifidus* at different rates of aeration, on successive days. The ordinate represents activity as a percentage of the maximum activity obtained.

○——○ 3 litres of air/litre of medium/minute.
×——× 2 „ „ „ „
z.....z 1 litre „ „ „
●—·—·● ½ „ „ „ „

and assembled in essentially the same manner. Air from the compressor was sterilized by passing through a cotton-wool filter, moistened by bubbling through sterile water, and distributed via a manifold to the bottles. It was later found preferable to have an individual filter on each bottle, so that the water used for moistening the air need not be sterile.

Medium. Five per cent malt extract medium was used for the experiments.

Preparation of the inoculum.

Since the inoculum was added through a comparatively narrow tube it had to be both finely divided and suspended in liquid. For comparison with surface culture methods the following procedure was used:

Malt agar plates were poured in the usual manner and, when cool, the surface was covered with a sterile cellophane disc. The fungus grew readily on the cellophane, and, when mature, could be scraped away with a sterile scalpel. It was transferred to a sterile bottle containing glass beads and distilled water. This was shaken vigorously for 10 to 15 minutes on a mechanical shaker, at the end of which time the liquor was smooth and milky.

A simpler way of preparing the mycelial suspension was as follows: The fungus was grown on liquid medium in 20-oz flat bottles containing glass beads. When the mycelium was well grown the cotton wool plugs were replaced by sterile caps and the bottles shaken violently. Agar slope cultures were found to be unsuitable for this method of preparation.

In vigorously aerated submerged cultures the mycelium was in a suitable form for use as inoculum with further preparation, and this was used for the larger scale work.

The effect of different rates of aeration.

Five 1-litre bottles, each containing 700 ml. of a 5 per cent malt extract medium and inoculated with 10 ml. of a mycelial suspension, were used for these observations. The air flow in the various bottles was adjusted to 3, 2, 1 and ½ litres of air per litre of medium per minute and, in the fifth bottle, to a very slow trickle. One ml. of sterile tri-amyl-citrate was added to each bottle to prevent frothing.

RESULTS.

Activity.

The maximum yield was attained more rapidly in the more vigoruosly aerated cultures, but after 12 days was approximately equal in the bottles aerated with 1, 2 and 3 litres per litre per minute. The experiment was terminated after 14 days, by which time the glass tubes became blocked by mycelium. The activity in the bottle aerated with half a litre per litre per minute was still considerably less than in the other bottles. No activity was demonstrated in the bottle aerated by a slow trickle (Fig. 1–7).

Similar results were obtained with 10-litre bottles. Cultures aerated with a quarter of a litre per litre per minute grew well and blocked the tubing within 12 days, but the culture fluid remained inactive. Cultures aerated with 1 litre per litre per minute grew well and activity was demonstrated after 8 days, but mycelium eventually blocked the tubing.

It is clear that antibiotically active culture fluid can be readily obtained in submerged culture of *Coprinus quadrifidus*. It was somewhat difficult to separate the fluid from the spongy mass of mycelium, and it was necessary to squeeze it out in a press.

SUMMARY.

Coprinus quadrifidus (Peck.) was grown in stationary surface culture in large vessels and in submerged aerated liquid culture, during which time its antibiotic

activity was developed. Good results were obtained in surface culture on 2·5 per cent malt extract medium at a depth of 2 cm. incubated at 24° C. Inoculation was from agar plates incorporating granulated cork. The agar was finely divided and inoculated with a long metal shovel into the large vessels. Good activity was obtained in submerged culture when the rate of aeration was over 1 litre per litre per minute when a 5 per cent malt extract medium was used. Inoculation was made by pipette with a mycelial suspension of the culture in distilled water.

I am indebted to Professor Sir Howard Florey, Dr. M. A. Jennings and Dr. K. Crawford for help in the preparation of this paper. I wish to thank Mr. J. Kent for technical assistance, Mr. Axtell and Mr. A. Graham for the photographs and Miss W. M. Poynton for secretarial assistance.

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IMMUNOLOGICAL ASPECTS OF THE THYMOL-TURBIDITY REACTION.

J. R. MARRACK, R. G. S. JOHNS AND H. HOCH.

From London Hospital Medical College.

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In the thymol turbidity test of liver function, introduced by Maclagan (1944), a saturated solution of thymol in barbiturate buffer of pH 7·8 is added to a small volume of the serum to be tested. With serum derived from a patient suffering from disease of the parenchyma of the liver or from certain other diseases (Maclagan, 1946), the mixture becomes more turbid than it does with normal serum and floccules settle out in 24 hours. The amount of floccules formed is of the order of 15 mg. per 1 ml. of serum; they consist of about 1/3 thymol, 1/3 phospholipid and cholesterol and 1/3 protein (Maclagan, 1944).

The main difference between the electrophoretic patterns of the original serum and of the clear supernatant liquid after the floccules have settled is that the ratio of β-globulin to (albumin + α_1-globulin) is reduced; the ratio of γ-globulin to (albumin + α_1-globulin) also may be reduced, but to a less degree (Cohen and Thompson 1947; Kunkel and Hoagland, 1947). These patterns indicate that the material in the floccules comes mainly from the β-globulin, although the γ-globulin may contribute a part. Cohen and Thompson also found that the floccules, resuspended in barbiturate buffer of pH 8·6, had the mobility of β-globulin.

However, MacLagan and Bunn (1947) and Kunkel and Hoagland (1947) have produced good evidence that γ-globulin plays the most important part in the reaction. Kunkel and Hoagland resuspended the floccules in barbiturate buffer of pH 8·5 containing 1 per cent of the detergent "tween," which was said not to affect the electrophoretic pattern of normal serum; a clear solution was obtained. With further treatment to remove the lipid and "tween" a solution was obtained of a protein that migrated extremely slowly. It seemed that as lipid was removed the remaining protein migrated more slowly. Kunkel and Hoagland suggested that some β-globulin may represent a light combination of lipid and γ-globulin. If this were so it might be expected the antigenic γ-globulin would exceed that estimated from the electrophoretic pattern.

The division of the globulins of serum into the 4 components α_1-, α_2-, β- and γ-globulin depends on a single property—the mobility in an electric field. There is some evidence that suggests that globulin that is not included in the electrophoretic γ-globulin may form precipitates with antisera to γ-globulin. Also γ-globulin is a family of proteins with similar mobilities; it is possible that the members of this family differ antigenically. Deutsch, Alberty, Gosting and Williams (1947) have separated human γ-globulin into two fractions, γ_1- and γ_2-

globulin. Their results suggest that these two fractions differ antigenically. It is therefore necessary to consider how far immunological methods can be used to detect and measure γ-globulin. We will call γ-globulin, as measured or isolated by electrophoresis, electrophoretic γ-globulin, and globulin that resembles γ-globulin in its behaviour as antigen, antigenic γ-globulin.

METHODS.

Sera, supernatant fluids and preparations of γ-globulin were examined by electrophoresis in phosphate buffer of pH 8 and ionic strength 0·1. The patterns were analyzed by the method of Pedersen (1940) (see also Hoch-Ligeti, Hoch and Goodall, 1949). Gamma-globulin was prepared from normal human serum by the method of Kendall (1937, 1938). This fraction is the globulin that is precipitated by 1/3 saturation with ammonium sulphate, and is not precipitated on dialysis against distilled water. Kendall called this fraction α-globulin at a time when the nomenclature of Tiselius was first coming into use ; we call these preparations Kα. Five samples were examined by electrophoresis (Fig. 1) ; in 3 ($K\alpha_4$, $K\alpha_6$ and $K\alpha_8$) γ-globulin made up over 96 per cent of the total protein. $K\alpha_8$ was the only sample in which two distinct γ-globulin fractions could be detected. $K\alpha_4$ and $K\alpha_5$ were used for immunizing rabbits, but not in the studies of reactions with the antisera.

Thymol turbidity reactions were done by Maclagan's (1944) method ; the turbidity was measured with a photoelectric instrument.

Floccules and supernatant fluid after separation of the floccules were obtained by the method of Cohen and Thompson (1947) from sera that gave high turbidity reactions. The thymol buffer reagent used was that of Maclagan ; the volume of reagent against which the mixture of serum and reagent was dialysed was never less than 60 times that of the serum used. This method is open to one of the objections raised by Maclagan and Bunn (1947), that the concentration of protein in the dialysing sac is much higher than in the mixture made in the ordinary test. Floccules formed in this way were washed 3 times with thymol-buffer and used for immunizing a rabbit and for extraction of the protein. For this extraction the floccules were rubbed up with saline and left for not less than 3 days, with repeated shaking at 4° C. ; on centrifuging slightly turbid extracts were obtained. These were dialysed against saline to remove residual thymol.

Rabbits were immunized by intramuscular injection of protein precipitated with alum according to the method of Proom (1943). However, the protein was freshly precipitated before each injection ; it was not precipitated in bulk and kept as a stock, as recommended by Proom. Two rabbits, " Grey " and " Tabby," were immunized with various sample of Kα, and one, " Persil," with floccules. For injection the floccules were suspended in saline and precipitated with alum. Injections were made at intervals of two weeks, and the rabbits were bled from an ear vein about 11 days after an injection. Antisera to Kα prepared in this way have proved very satisfactory for the Coombs-Mourant reaction—Coombs, Mourant and Race (1945). They can be used, without preliminary absorption, in dilutions of over 1 in 1000. The antisera obtained at different bleedings of " Grey " and " Tabby " differed in their contents of antibody that was not precipitated by Kα ; those used in this investigation contained minimal amounts of such antibodies to other protein fractions of serum.

Precipitation curves.

A constant amount of antiserum (usually 0·5 ml.) was placed in each of a series of centrifuge tubes. A series of progressively increasing amounts of a solution containing antigen was added to the tubes and the volume made up to a standard with saline. The tubes were kept at room temperature until floccules appeared in all, and then kept at 4° C. for 2 days. They were then centrifuged, the precipitates were washed 3 times with ice-cold saline, and the protein in them estimated by a modification of the method of Folin and Ciocalteu (Heidelberger and MacPherson, 1943 ; Heidelberger and Anderson, 1944). In the first of these

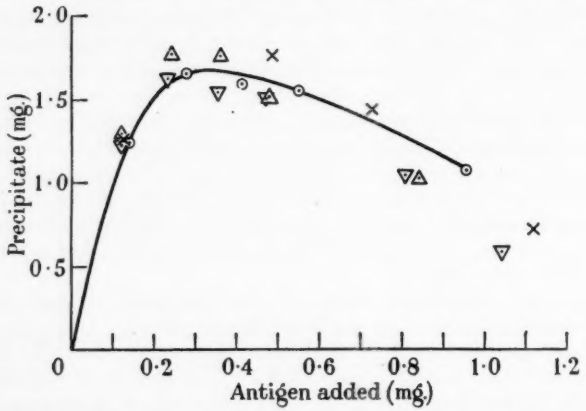

FIG. 2.—Precipitation curves given with antiserum ("Tabby" 23/11/48) to Kendall's α-globulin, by two preparations of Kendall's α-globulin ($K\alpha_7$, △ ; $K\alpha_8$, ▽) ; electrophoretic γ-globulin, ⊙ ; normal serum, ×.

papers no reference is made to the use of copper ; this is corrected in the second paper. The amount of antigen added and amount of precipitate formed were recalculated for 1 ml. of antiserum. The amount of precipitate from 1 ml. of antiserum was plotted, as ordinate, against the amount of antigen added, as abscissa (Fig. 2 and 3). When the protein of whole human serum was used as precipitating antigen the abscissae are the amounts of γ-globulin, estimated by electrophoresis, in the volumes of human serum added.

EXPLANATION OF PLATES.

FIG. 1.—Electrophoretic Patterns of Kendall's α-globulin.

	Sample.	Concentration (g./100 ml.).	Time of electrophoresis at 6·7 V/cm. : minutes. Ascending : (left).	Descending : (right).	Per cent of γ-globulin.
a	$K\alpha_4$	1·2	70	—	Over 96. No measurement of areas.
b and *c*	$K\alpha_5$	1·3	33	35	92·5. The two fast peaks are those of α- and β-globulin.
			81	79	
d	$K\alpha_6$	1·3	45	47	Over 96. No measurement of areas.
e	$K\alpha_7$	0·4	53	55	93. The small fast peak is that of β-globulin.
f	$K\alpha_8$	1·2	107	107	Over 96. No measurement of areas. Two γ-components.

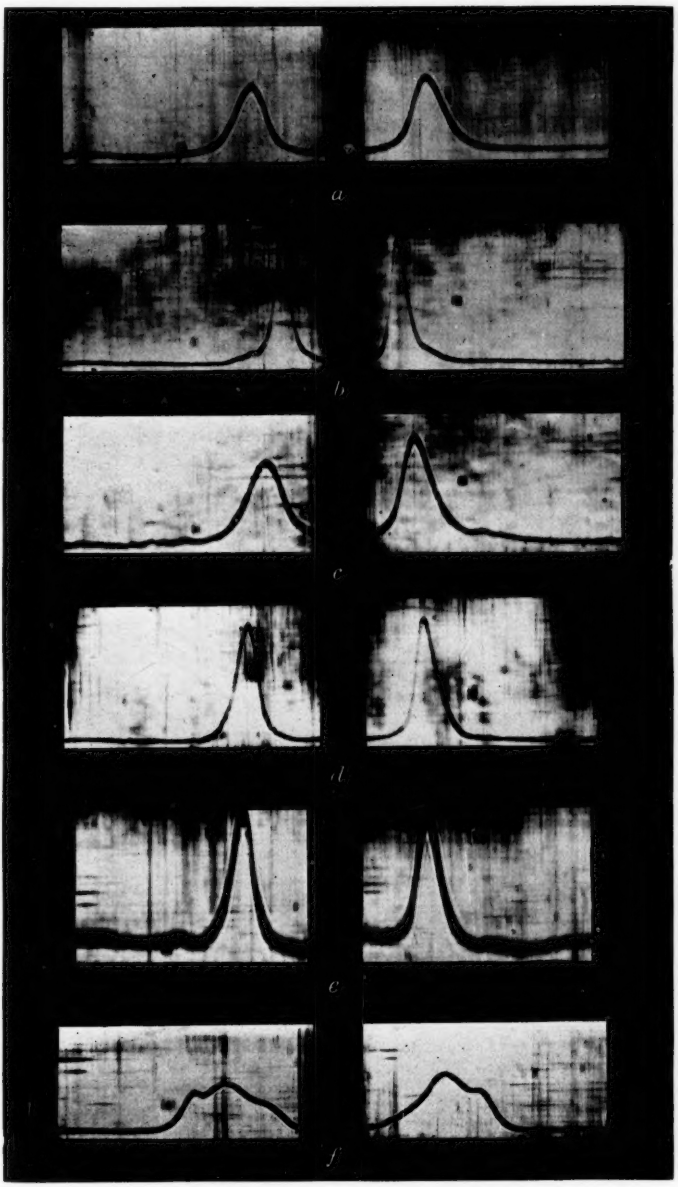

FIG. 1.

Marrack, Johns and Hoch.

Proteins, other than those in antigen-antibody precipitates, were estimated by Kjeldahl's method, using the factor 6·25.

Antisera to γ-Globulin.

The term γ-globulin is conventional; it is applied to a group of globulins whose mobility lies within a certain range. The γ-globulin prepared by Kendall's method is only a part of the total γ-globulin of serum; a considerable amount of γ-globulin is discarded in the fraction that is insoluble in distilled water. The differences between the curves given by $K\alpha_8$ and γ-globulin, separated by electrophoresis, with antiserum "Tabby" 23/11/48 (Fig. 2) are within the errors of the methods involved. If part only of the γ-globulin separated by electrophoresis had reacted with the antiserum as Kα did, more of this electrophoretic γ-globulin than of Kα would be needed to form a given weight of precipitate with 1 ml. of

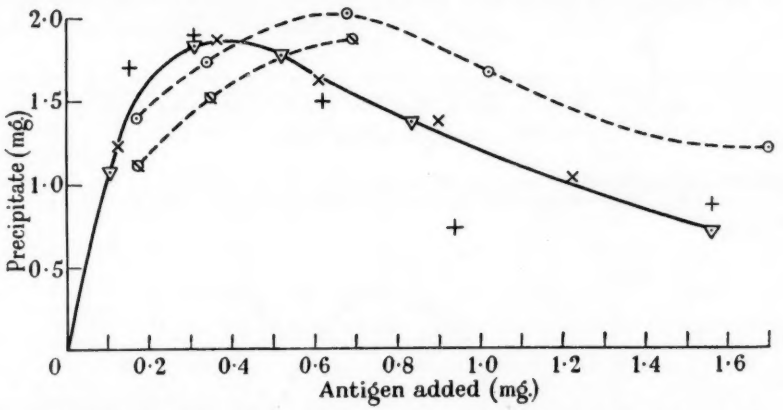

FIG. 3.—Precipitation curves given with antiserum ("Grey," 23/11/48) by a preparation of Kendall's α-globulin ($K\alpha_8$, ▽); normal serum, ×; serum of Mu, +; extracts of the thymol-floccules from the serum of Mu., ⊙ and ⊗.

antiserum, in the zone of antibody excess. It appears therefore that there is no difference, as antigen, between the Kα and other proteins of the electrophoretic γ-globulin. With this antiserum the curves given by the sample, $K\alpha_7$, which contained some β-globulin, and by diluted whole serum were slightly higher than that given by γ-globulin separated by electrophoresis and by $K\alpha_8$; that is, $K\alpha_7$ and whole serum precipitated slightly more antibody than the pure γ-globulin precipitated. Also whole serum formed a slight precipitate with the supernatant fluid left when all the antibody to $K\alpha_8$ had been precipitated by addition of an appropriate amount of $K\alpha_8$. This antiserum therefore contained a slight amount of antibody to serum proteins other than electrophoretic γ-globulin. There is no evidence of an antigenic difference between electrophoretic γ-globulin, the γ-globulin present in whole serum and the γ-globulin of either $K\alpha_7$ or $K\alpha_8$.

A very slight precipitate formed when normal human serum was added to antiserum "Grey" 23/11/48, which had been cleared of antibody to $K\alpha_6$; this antiserum, therefore, contained a very slight amount of antibody to protein

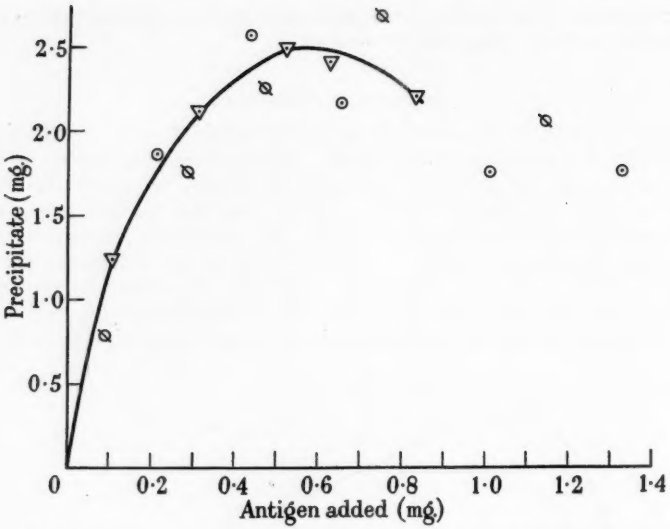

FIG. 4*a*.—Precipitation curves given with antiserum ("Grey," 28/10/48) by $K\alpha_s$, ▽; serum of Br., ⊙; and extract of the thymol-floccules from the serum of Br., ⊘.

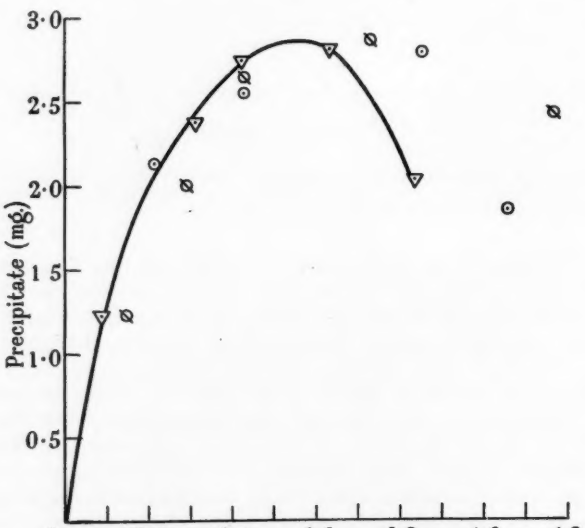

FIG. 4*b*.—Precipitation curves given with antiserum ("Grey," 28/6/48) by $K\alpha_s$, ▽; serum of Go., ⊙; and extract of the thymol-floccules from the serum of Go., ⊘

besides the anti-Kα. However, the precipitation curves given by $K\alpha_6$ and normal human serum with this antiserum were identical (Fig. 3). The normal serum did not contain more than traces of any globulin, besides the eletrophoretic γ-globulin that formed a precipitate with the antibodies to Kα. If it had done so the amount of electrophoretic γ-globulin in a volume of human serum that formed a given weight of precipitate with 1 ml. of this antiserum (in the zone of antibody excess) would have been less than the amount of Kα that formed the same weight of precipitate with 1 ml. of antiserum. The effect on the precipitation curve would be similar to that of reducing the scale of the abscissae.

The difference between the curves given by the sera of patients Go. and Br. and that given by $K\alpha_6$ (Fig. 4*a* and 4*b*) are not more than would be accounted for by the errors of the precipitation method and of the estimation of γ-globulin by electrophoresis. The curve given by the serum of Mu. (Fig. 3) suggests that the antigenic γ-globulin in this serum was about 30 per cent more than the electrophoretic γ-globulin ; but it is difficult to draw conclusions from this curve, as its shape differs from that of the curve given by $K\alpha_6$.

TABLE I.—*Amounts of α_2-, β- and γ-Globulin in Serum and in the Supernatant Fluid after Separation of the Thymol-floccules, as g. per 100 ml. of Original Serum, calculated on the assumption that the (albumin + α_1-globulin) is not changed.*

Patient.	Diagnosis.	Thymol-turbidity (units).	Total protein (Se.).	α_2-globulin.		β-globulin.		γ-globulin.		Difference Se–Su : β+γ-globulin.
				Se.	Su.	Se.	Su.	Se.	Su.	
Bo.	Cirrhosis liver	20	5·5	0·3	0·25	1·64*	1·56*	2·05†	1·46†	0·67
Go.	Subacute hepatitis	20	5·2	0·5	0·25	1·00	0·36	1·82	1·50	0·96
Br.	Acute hepatitis	16	6·2	0·43	0·405	1·16	0·65	2·14	2·08	0·57
Mu.	Rheumatoid arthritis	14	9·25	0·67	0·69	1·13	0·72	3·85	3·55	0·71

Se, serum ; Su, supernatant.
* γ_1-globulin included with β-globulin in this fraction.
† γ_2-globulin only.

The Protein Precipitated by the Thymol-buffer Reagent.

Electrophoresis.

The electrophoretic patterns of four supernatant fluids after separation of the floccules were compared with the patterns of the original sera. In Table I the amounts of the other components left in the supernatant fluids, per 100 ml. of the original serum, are calculated on the assumption that no (albumin + α_1-globulin) was precipitated. In 3 cases the biggest reduction was that of the β-fraction. In 2 of these, Go. and Mu., the γ-fractions also were reduced ; in the case of Br. this apparent reduction was within experimental error. In the 4th case (Bo.) it was not possible to demarcate the peaks of β- and γ_1-globulin from each other and the demarcation between γ_1- and γ_2-globulin was uncertain ; it seems that the main reduction was that of the γ_2-globulin.

If all the reduction of the β- and γ-globulin areas in case Mu. were due to precipitation of protein the total precipitate per ml. of serum would be 7·1 mg., of which 4·1 mg. would be β-globulin and 3·0 mg. would be γ-globulin. Actually

3·7 mg. (3·54 mg. in the extracts and 0·2 mg. unextracted) of protein was found in the floccules per ml. of serum used. This is equal to the apparent reduction of γ-globulin within the limits of error of electrophoretic estimations. The apparent loss of β-globulin in this case and in case Go. may have been due to loss of lipid that migrates with the β-fraction.

Immunological.

Eighty per cent of the protein in the floccules from serum Mu. was extracted in the first extract and 7 per cent in the second extract. The precipitation curves given by these extracts with serum " Grey " 23/11/48 are given in Fig. 3 ; the abscissae are the total amounts of protein added per 1 ml. of antiserum. The first curve would be given if about 75 per cent of the protein were γ-globulin, and if some of the remaining protein would form a precipitate with the residual antibody of the antiserum. The second curve would be given by a solution containing about 50 per cent of γ-globulin. So that it seems that some 30 per cent of the protein in these floccules was not γ-globulin, but it is possible that some of the protein had been denaturated by prolonged exposure to thymol. The curves given by the extracts of the floccules from the sera of Br. and Go. (Fig. 4*a* and 4*b*) would fit the curves given by $K\alpha_6$ with the same antiserum if these extracts contained about 80 per cent of γ-globulin.

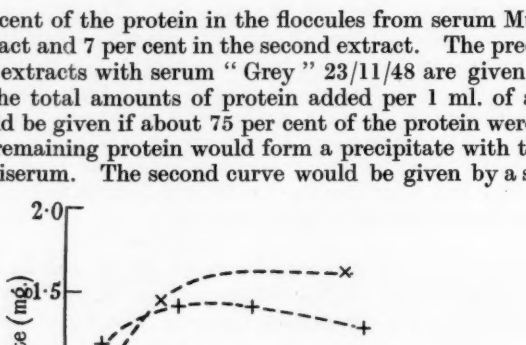

FIG. 5.—Precipitation curves given with antiserum to thymol-floccules (" Persil," 26/3/48) by $K\alpha_6$, ▽ ; and by two normal sera, + and ×.

Antiserum to Floccules.

The curves given by normal serum with antiserum from a rabbit immunized with the floccules (Fig. 5) were considerably higher than that given by Kα. Human serum still formed considerable precipitate with this antiserum after all the antibody to Kα had been precipitated. About 70 per cent of the total antibody was antibody to γ-globulin. Immunization with the floccules gives rise to antibodies to γ-globulin, together with lesser amounts of antibodies to other fractions of the serum protein.

DISCUSSION.

These experiments afford no evidence of the presence in normal serum of a globulin which is antigenically identical with electrophoretic γ-globulin, but has a mobility greater than that of γ-globulin; nor of antigenic differences between whole γ-globulin and the fraction separated by Kendall's (1937) method. However, Kendall used antisera to the antigen (Kendall's α-globulin) that we used for estimating the amount of this globulin in serum. He absorbed the antiserum with a preparation of other serum globulins in order to remove antibodies to other antigens; but the amounts of this fraction that he found by this means in normal serum were nearly twice the amounts of γ-globulin that are found by electrophoresis. Also, Jager, Smith, Nickerson and Brown (1948) immunized with fractions II-1, 2, Squibb 324–329 and II-3, Squibb, 341; no proteins faster than γ-globulin were detected by electrophoresis in these fractions. They used the antisera obtained, without removing possible antibodies to other globulin fractions, for estimating γ-globulin. The amounts that they found in normal sera were nearly twice the amounts found by electrophoresis. These antisera contained no antibodies to serum albumin, but it is possible they contained antibodies to globulin fractions which are not antigenically similar to γ-globulin. For Deutsch and colleagues (1947) found that antisera to γ_2-globulin reacted with a sample of serum albumin that contained no γ-globulin, detectable by electrophoresis, and less than 2 per cent of α-globulins; and sera from our rabbits "Grey" and "Tabby" after further immunization with Kα contained antibodies to serum albumin, although no albumin could be detected by electrophoresis in our preparations of Kα. Conversely, Coombs and Mourant (1947) found that sera of rabbits immunized with albumin, prepared by Cohn's methods, contained antibodies to γ-globulin. Jager and colleagues (1948) suggest that their sera cross-reacted with other fractions of serum globulin, antigenically similar to γ-globulin. Such a cross-reaction would be similar to that of β-globulin of horse serum with antisera to the globulin of the horse-antipneumococcal antibody, which was a γ-globulin (Treffers, Moore and Heidelberger, 1942). Some of the antisera from our rabbits "Grey" and "Tabby" reacted with fractions of serum globulin prepared by electrophoresis that were free from γ-globulin. The character of the reaction resembled that of a cross-reaction. These sera were not used in this investigation. A distinction is drawn between (1) a *cross-reaction* between an antibody and a substance similar to the homologous antigen, and (2) a reaction of a substance, which is present as an impurity in the preparation of antigen used for immunization, with the antibodies which it provokes. The reaction of gum acacia with the purified antibody to Type II pneumococcal polysaccharide (Heidelberger, Avery and Goebel, 1929) is a cross-reaction. The pneumococci used for immunization do not contain gum acacia; but the gum acacia resembles the pneumococcal polysaccharide, and precipitates part of the antibody that is precipitable by the pneumococcal polysaccharide (Marrack and Carpenter, 1938). The formation of floccules when a heterologous cross-reacting antigen is added to antiserum is much slower than it is when the homologous antigen is added.

Our experiments, on the whole, support the conclusions of Maclagan and Bunn (1947). The greater part of the protein precipitated in the floccules reacted like γ-globulin with antisera to γ-globulin. In cases such as Go. and Mu. the

γ-globulin area in the supernatant fluid after flocculation was reduced relatively to the albumin area. The difficulty lies in explaining cases such as Br. in which the reduction in the γ-globulin area was negligible, but the floccules contained a protein that reacted with antiserum as γ-globulin did. In this case there was no evidence that the antigenic γ-globulin in the serum exceeded the electrophoretic γ-globulin.

SUMMARY.

(1) Antisera were obtained by immunizing rabbits with γ-globulin, prepared by Kendall's (1947) method. The sera used contained minimal amounts of antibody that reacted with other proteins of serum.

(2) No evidence was found of antigenic differences between the γ-globulin prepared by Kendall's method, γ-globulin prepared by electrophoresis and the γ-globulin as present in normal serum.

(3) No evidence was found of the presence, in the serum of two patients with liver disease, of a globulin which had a mobility greater than that of γ-globulin, but reacted antigenically as γ-globulin; the evidence of the presence of such globulin in the serum of a patient with rheumatoid arthritis was doubtful.

(4) The protein extracted from the floccules formed in the thymol-flocculation reaction reacted with antiserum to γ-globulin as it would if 70 or more per cent of it were γ-globulin.

(5) The principal antibody in the serum of a rabbit immunized with the floccules formed in the thymol-flocculation reaction was antibody to γ-globulin.

(6) The reduction of the γ-globulin of sera in this reaction, as estimated from the electrophoretic patterns of the serum and supernatant fluid after flocculation, was, in one out of three cases, too little to account for the γ-globulin found in the floccules.

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VIRUS EXALTATION.

G. M. FINDLAY AND E. M. HOWARD.

From the Pharmacological Laboratories, School of Pharmacy, London.

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WHEN two viruses attack the same host at the same time one of three things may happen. The two viruses may develop independently: such independent growth has been recorded in animal tissues by Syverton and Berry (1947) in relation to vaccinia, herpes, and virus B, and in plants by McWhorter and Price (1949), who found that tobacco mosaic and tobacco etch viruses developed independently in the same cell. One virus may interfere with the growth of the other. Local interference with the growth of one virus by another was first noted by Jenner (1804), who pointed out that vaccinia would not give a typical lesion on skin infected by herpes simplex. Magrassi (1935 *a* and *b*) drew attention to the fact that implantation of a non-encephalitogenic strain of herpes simplex virus on the conjunctiva of the rabbit prevented the development of encephalitis when an encephalitogenic strain of herpes was later implanted on the same conjunctiva. It is by no means certain that this localized phenomenon is identical with the generalized interference phenomenon observed by McKinney (1929) with plant viruses, and by Hoskins (1935) with neurotropic and viscerotropic strains of yellow fever virus. Findlay and MacCallum (1937) showed that interference could occur between viruses which were not antigenically related.

The third possibility when two viruses are inoculated into the same host is that one virus may exalt the virulence of the other. Vanterpool (1926) showed that mild tobacco mosaic and potato mottle viruses when inoculated into the same host, the tomato, cause a severe disease known as streak. The tick *Ixodes ricinus* is frequently the carrier of two viruses, tick-borne fever virus and louping ill virus, both of which are pathogenic for the sheep. These viruses are quite distinct (Gordon, Brownlee and Wilson, 1932; Gordon, Brownlee, Wilson and MacLeod, 1932; MacLeod and Gordon, 1932, 1933), but when louping ill alone is injected into the sheep it does not invariably cause encephalomyelitis; if tick-borne fever virus is also injected, encephalitis almost invariably occurs. More recently Lépine and Marcenac (1948) found that when herpes simplex virus and pseudorabies were used to infect the same mouse the virulence of the latter virus was increased some fifty times whether the two viruses were injected simultaneously or whether the inoculation with herpes simplex was delayed till some days after that of the pseudorabies virus. On the other hand the virulence of the herpes virus was not increased by sublethal dilutions of the pseudorabies virus.

EXALTATION OF COLUMBIA SK VIRUS BY OTHER VIRUSES.

A somewhat similar phenomenon was discovered accidentally in attempting to demonstrate interference between mouse adapted strains of poliomyelitis and

Columbia SK virus. The latter virus was originally obtained by Jungeblut, Sanders and Feiner (1942) during attempts to adapt a human poliomyelitis virus (Y–SK) to mice. There is general agreement that Columbia SK virus is a rodent encephalomyelitis virus and that it is antigenically related to the Mengo encephalomyelitis virus; it thus belongs to the group of encephalo-myocarditis viruses. It is pathogenic for cotton rats, mice, guinea-pigs, hedgehogs, and in later passages for monkeys. It is highly pathogenic in mice by the intracerebral, intranasal, intraperitoneal, and intravenous routes. It also infects when fed by mouth and when injected intramuscularly into very small mice (Rustigan and Pappenheimer, 1949): in very high dilutions it causes paralysis, but in lower dilutions the mice die in from 36 to 48 hours with weakness and collapse. When mice are injected intraperitoneally with 0·2 ml. of falling dilutions of mouse brain infected with Columbia SK the LD 50 is found in different experiments to be from 10^{-6} to 10^{-7}.

The following experiments show the exalting effect of the M.E.F. 1 strain of mouse-adapted poliomyelitis virus on Columbia SK virus in mice.

TABLE I.—*Experiment* 1.

Number of days to death. (S = survival.)

Columbia SK 10^{-6} and M.E.F. 1 10^{-1}	Columbia SK 10^{-6}	M.E.F. 10^{-1}	
4	8	S	S
5	11	S	S
5	S	S	S
7	S	S	S
Columbia SK 10^{-7} and M.E.F. 1 10^{-1}	**Columbia SK 10^{-7}**		
5	13		
5	S		
6	S		
7	S		
Columbia SK 10^{-8} and M.E.F. 1 10^{-1}	**Columbia SK 10^{-8}**		
5	6		
5	S		
5	S		
7	S		
Columbia SK 10^{-9} and M.E.F. 1 10^{-1}	**Columbia SK 10^{-9}**		
8	S		
8	S		
9	S		
S	S		
Columbia SK 10^{-10} and M.E.F. 1 10^{-1}	**Columbia SK 10^{-10}**		
8	S		
8	S		
9	S		
14	S		

Experiment 1.

Mice were injected intraperitoneally with 0·2 ml. of a 1 in 10 suspension of mouse cord infected with M.E.F. 1 strain of poliomyelitis virus; 3 hours later the mice were injected intraperitoneally with 0·2 ml. of mouse brain infected with Columbia SK virus and diluted to form suspensions of 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-11}; other control mice were given M.E.F. 1 virus intraperitoneally (Table I).

Further experiments gave a similar result, as shown by the protocols in Table II.

TABLE II.—*Experiments* 2, 3 *and* 4.

Number of days to death. (S = survival.)

Experiment 2.

Columbia SK 10^{-8} and M.E.F. 1 10^{-1}	Columbia SK 10^{-8}	M.E.F. 1 10^{-1}	
5	13	S	S
10	19	S	S
19	S	S	S
S	S	S	S
Columbia SK 10^{-9} and M.E.F. 1 10^{-1}	**Columbia SK 10^{-9}**		
12	14		
15	15		
16	S		
S	S		
Columbia SK 10^{-10} and M.E.F. 1 10^{-1}	**Columbia SK 10^{-10}**		
10	12		
12	S		
14	S		
S	S		

Experiment 3.

Columbia SK 10^{-9} and M.E.F. 1 10^{-1}	Columbia SK 10^{-9}	M.E.F. 1 10^{-1}	
8	S	S	S
9	S	S	S
10	S	S	S
S	S	S	S
Columbia SK 10^{-10} and M.E.F. 1 10^{-1}	**Columbia SK 10^{-10}**		
7	S		
9	S		
S	S		
S	S		
Columbia SK 10^{-11} and M.E.F. 1 10^{-1}	**Columbia SK 10^{-11}**		
9	S		
S	S		
S	S		
S	S		

TABLE II—*continued.*

Experiment 4.

Columbia SK 10^{-7} and M.E.F. 1 10^{-1}		Columbia SK 10^{-7}	M.E.F. 1 10^{-1}
8		14	S S
8		15	S S
8		S	S S
9		S	S S

Columbia SK 10^{-8} and M.E.F. 1 10^{-1}	Columbia SK 10^{-8}
8	14
8	S
8	S
9	S

Columbia SK 10^{-9} and M.E.F. 1 10^{-1}	Columbia SK 10^{-9}
9	7
11	14
14	S
16	S

Columbia SK 10^{-10} and M.E.F. 1 10^{-1}		Columbia SK 10^{-10}	
7	9	14	S
8	10	S	S
9	10	S	S
9	14	S	S

When the same experiment was carried out substituting a suspension of normal mouse cord for the M.E.F. 1 cord there was no enhancement. When the injection of Columbia SK virus was delayed 24 hours instead of 3 hours, 2 mice died in the group given Columbia SK virus 10^{-9} in 5 and 7 days; no mice died in the groups given Columbia SK virus 10^{-10} and 10^{-11}; control mice showed no deaths. The exalting effect was obtained with M.E.F. 1 cord diluted 1 in 100, to a slight extent when diluted 1 in 200 but not when diluted 1 in 400.

VIRUS EXALTATION OF COLUMBIA SK VIRUS BY WATT VIRUS.

An attempt to determine whether the exaltation of Columbia SK virus was specifically related to M.E.F. 1 poliomyelitis virus was made, employing Watt virus. The Watt virus was recovered from two mice which were inoculated intraperitoneally with stools from a child diagnosed clinically as non-paralytic poliomyelitis. The evidence at present available suggests that this virus is a strain of mouse encephalomyelitis virus and unrelated to the Columbia SK virus, but further investigations are proceeding. The Watt virus does not normally infect when injected intraperitoneally in mice.

Mice were inoculated intraperitoneally with 0·2 ml. of a 1 in 10 mouse hind brain and cord infected with Watt virus; 3 hours later the mice were inoculated with 0·2 ml. of dilutions of mouse brain infected with Columbia SK virus 10^{-6}, 10^{-7}, 10^{-8}; controls were inoculated intraperitoneally with 10^{-1} Watt virus alone and with Columbia SK virus 10^{-6}, 10^{-7} and 10^{-8} (Table III).

TABLE III.—*Experiment* 5.

Number of days to death. (S = survival.)

Columbia SK 10^{-6} and Watt 10^{-1}	Columbia SK 10^{-6}	Watt 10^{-1}
5	8	S
5	11	S
7	S	S
7	S	S
		S
		S
		S
		S
Columbia SK 10^{-7} and Watt 10^{-1}	Columbia SK 10^{-7}	
6	13	
6	S	
7	S	
14	S	
Columbia SK 10^{-8} and Watt 10^{-1}	Columbia SK 10^{-8}	
6	6	
8	S	
9	S	
S	S	

A similar result was obtained with the Columbia SK virus and the Watt virus as with the M.E.F. 1 virus. The exaltation of Columbia SK virus does not therefore appear to be specifically associated with a particular neurotropic virus.

Instead of using mouse brain infected with living M.E.F. 1 or Watt virus mouse brain suspension M.E.F. 1 virus heated to 56° C. for 20 minutes was used. No stimulating effect was found with this heated virus.

TABLE IV.—*Pathogenicity of Columbia SK Virus Injected Intracerebrally into Mice* (0·03 *ml. Injection*).

Dilution of virus.	Number of days till death.
10^{-4}	2, 2, 2, 3
10^{-5}	2, 2, 3, 4
10^{-6}	2, 3, 3, 4
10^{-7}	2, 2, 3, 4
10^{-8}	3, 3, 3, 7
10^{-9}	3, 6, 8, 8
10^{-10}	14, 14, 15, 15
10^{-11}	3, 8, 11, 15
10^{-12}	14, 15, S, S
10^{-13}	15, S, S, S

DISCUSSION.

Interference between viruses whether antigenically related or not can be explained on the theory that the viruses compete either for groups on the surface of susceptible cell or for metabolites within the cell. Virus exaltation is less

easy to explain. It is noteworthy, however, that in the three cases where virus exaltation has been seen, the viruses which have had their pathogenicity increased are all ones which have first a haematogenous phase, after which they may or may not localize in the central nervous system. The action of the exalting virus appears to facilitate the localization of the exalted virus in the central nervous system. A study of the effects of Columbia SK virus inoculated intracerebrally in a dose of 0·03 ml. shows that the LD 50 is 10^{-12} (Table IV), which corresponds closely with the effects of intraperitoneal injection of Columbia SK virus and M.E.F. 1 virus. It may also be noted that whereas dilutions of 10^{-2} or 10^{-3} kill the mice in two to three days without any specific nervous symptoms, the dilutions of 10^{-9} onwards almost always cause paralysis of the legs. In addition, when suspensions of M.E.F. 1 infected brain are first inoculated intramuscularly there is no exalting action on the Columbia SK virus subsequently injected intraperitoneally. It is therefore suggested that the action of M.E.F. 1 virus is to facilitate spread of the Columbia SK virus to the central nervous system.

SUMMARY.

An example of virus exaltation in the mouse is described. Columbia SK virus injected intraperitoneally into mice in dilutions which are not normally lethal or which kill only after a long interval has its virulence increased by a previous intraperitoneal inoculation of M.E.F. 1 poliomyelitis virus or Watt mouse encephalomyelitis virus. Consideration of the three instances now known of exaltation of animal viruses shows that they each involve a neurotropic virus, the localization of which in the central nervous system is preceded by a period of blood spread. Exaltation would seem to consist in facilitating the passage of the virus across the blood-brain barrier.

We are most grateful to Professor G. A. H. Buttle, in whose laboratories these investigations were carried out. One of us (G.M.F.) is in receipt of a grant for expenses from the Medical Research Council, for which we desire to express our thanks.

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EXCRETION OF SPECIFIC ANTIGEN IN THE URINE IN TSUTSUGAMUSHI DISEASE (SCRUB TYPHUS).

J. L. O'CONNOR and JEAN M. MACDONALD.

From the Virus and Rickettsial Section, Commonwealth Serum Laboratories, Melbourne, N.2, Australia.

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VARIOUS workers have demonstrated the presence of rickettsial antigen in serum and in urine from cases of epidemic louse-borne typhus fever. Drobyshevskaya and Smorodintzeff (1944) showed the presence of circulating antigen in the serum by means of the complement-fixation test in 1942, and used this method for the early diagnosis of louse-borne typhus. This finding was confirmed by Léon (1942). Later Smorodintzeff and Fradkina (1944) absorbed the antigen in the serum onto carmine particles, rendering them agglutinable by typhus-immune serum. Léon (1942), using the precipitin test, showed the presence of epidemic typhus antigen in the urine, and employed this as a means of obtaining early laboratory diagnosis of the disease. At about the same time Fleck (1947), working in a prison camp in Poland, was also finding antigen in the urine of classical typhus cases, and was able to cause the production of Weil-Felix antibodies in rabbits with his preparations.

Using animals experimentally infected with Tsutsugamushi disease, Smadel, Rights and Jackson, in 1946, demonstrated the presence of soluble rickettsial antigen capable of fixing complement in the body fluids and in tissue extracts.

The work of Keogh, North and Warburton (1947, 1948), and Warburton, Keogh and Williams (1949) on the phenomenon of the coating of erythrocytes by antigens prepared from *Haemophilus influenzae* and other Gram-negative organisms, enabled them to develop a simple and reliable test for the diagnosis of influenzal meningitis, based upon the detection of antigen in the cerebro-spinal fluid. The investigation now being reported results from the application of their technique to the estimation of rickettsial antigen in the urine of patients suffering from scrub typhus. Preliminary tests had indicated that the method was more sensitive than either Léon's precipitin test or the carmine agglutination method of the Russian workers.

Through the courtesy of Dr. Joseph E. Smadel and his associates, of the Division of Virus and Rickettsial Diseases, Army Medical Department Research and Graduate School, Washington, D.C., U.S.A., we were able to make full use of the clinical material and records gathered by these workers during their field trials of chloromycetin in Malaya. The senior author is also greatly indebted to Dr. R. Lewthwaite, Director of the Institute of Medical Research, Kuala Lumpur, Malaya, for permission and facilities to carry out a considerable part of the investigation at that Institute, and for his interest in the work.

MATERIALS AND METHODS.

Chicken cells.

Blood was collected from the wing vein of chickens of the age of about six months direct into the modified Rous-Turner solution described by Simmons and Graydon (1945). Cells were washed twice and stored as a 25 per cent suspension in this solution at 4° C. Under these conditions, they remain in a completely satisfactory state, if sterile, for 6 to 7 days. Sensitized cells similarly preserved retain their characteristics unchanged for at least 4 days. We have not so far tested the effect of Alsever's solution. Human erythrocytes of Group O have also been tried, but agglutination takes twice as long to become complete, and the agglutination patterns are not so well defined.

Urine.

In nearly every case, the specimens of urine examined were those passed on waking in the morning, as Léon's investigations had shown a significantly higher proportion of positive results with 24-hour specimens than with single specimens. After collection, they were immediately stored at −20° C. until they could be examined. All specimens examined were free of micro-organisms.

For the test, the specimen, after thawing, was placed in a boiling water bath for 30 minutes, then cooled, filtered and examined at once. This procedure was used to destroy a haemagglutinin which appears to be excreted intermittently by most of the human subjects, both healthy and infected, whose urines were examined.

Serum.

The standard antiserum employed in all tests was a lot obtained from a convalescent human case of scrub typhus. It had a titre of 2560 against *Proteus OXK*. The same batch of serum was used throughout the investigation. Bulk lots of the working dilutions were prepared each morning and kept on ice during the day.

Polysaccharide preparations.

The method of extracting the active polysaccharide from *Proteus XK* is a modification of that described by Boivin in 1933 for other Gram-negative bacteria, and by de Burgh (1946) for *Proteus XK*. The details of some of its serological and other characteristics will be the subject of a separate report, but it is sufficient to say now that the fraction that has been used has the same immunological relationship to *Richettsia orientalis* as the X factor of *Proteus X*19, described by Castañeda (1934), has to *R. prowazeki*.

Our preparations have been made as follows: *Proteus XK* is grown for 7 to 8 hours at 37° C. in tubes of Mueller's broth, and nutrient agar in Roux flasks then is seeded with the young growing culture allowing 2 to 3 ml. of broth per flask. The inoculated flasks are incubated overnight at 37° C. and the next morning the growth is washed off with a minimum quantity of normal saline. The bacteria are washed twice with saline and then resuspended in M/4 trichloracetic acid, allowing 3 ml. of acid per Roux flask. The mixture is agitated in the cold room for 3 hours and then centrifuged. A further extraction with trichloracetic acid may be carried on to improve the yield if required, and the two supernatants pooled.

The liquid extract is made neutral to litmus with strong sodium hydroxide solution. Two volumes of absolute ethyl alcohol are then added, and sufficient saturated aqueous solution of sodium acetate to give a final concentration of 0·7 per cent by volume. Flocculation is allowed to occur in the cold room overnight. The precipitate is collected, dissolved in N/50 sodium hydroxide solution, allowing approximately 0·5 ml. per milligram of precipitate, and the solution is heated at 100° C. for 1 hour. Following this treatment, the mixture is cooled, neutralized with N/5 hydrochloric acid, and then centrifuged. The deposit is discarded, and to the supernatant is added 2 volumes of absolute ethyl alcohol and enough saturated aqueous solution of sodium acetate to give a final concentration of 0·7 per cent by volume. The mixture is allowed to stand overnight in the cold room, and on the following morning the precipitate is collected, thoroughly washed with ethyl alcohol, then with sulphuric ether, and finally dried.

The white fluffy powder so obtained is dissolved in distilled water to give a concentration of 1/1000. The solution so obtained should be opalescent and somewhat viscid. It is thoroughly shaken with $1\frac{1}{2}$ volumes of sulphuric ether for 15 minutes to remove a haemolysin usually present. After separation of the aqueous and ether phases is complete, the former is removed and distributed into ampoules for lyophilization. If kept sterile, and stored in the cold, this preparation appears to keep its activity unimpaired for at least one year—the longest period over which it has been observed. The yield obtained averages about 6 per cent of the dry weight of the whole organism.

The antigen so prepared produces satisfactory specific antibodies when injected into rabbits.

Much purer preparations have been made by careful repeated fractionation and electrophoresis, but are not required except for purposes of standardizing the final titrating system.

The antigen has also been extracted using the phenol method as modified by Warburton *et al.* (1949), and by the antiformin method of Castañeda (1934), but the method described above appears to give the most satisfactory yield of active material.

For use the dried antigen is dissolved in physiological saline containing 0·5 per cent formalin.

Standardization of reagents.

For convenience the techniques of Warburton *et al.* (1949) are summarized here. Some minor modifications have been introduced.

All dilutions were made using graduated pipettes, the unit volume used being 0·05 ml. All tests were performed in test tubes 75 mm. long and 10 mm. internal diameter, and all reactions allowed to proceed at room temperature.

To titrate the activity of the polysaccharide, serial two-fold dilutions of it were prepared in physiological saline commencing with a dilution of 1/1000. To nine volumes of each dilution was added one volume of packed washed fowl erythrocytes. After having been well mixed and allowed to stand for 15 minutes the cells were re-sedimented, washed twice, and then made up to a 5·0 per cent suspension in physiological saline. Each sample of cells was then titrated for degree of sensitization by adding one volume of the suspension to ten volumes of antiserum in a dilution of 1/200. The highest dilution of polysaccharide

which rendered the red cells completely agglutinable was taken as the activity of the preparation. The titre of the preparation used throughout the investigation was found to be 1,024,000.

For reading all other titrations, the pattern method was adhered to, but following densitometric estimations, factors were calculated to reduce all end-points to that equivalent to 50 per cent agglutination. Titres so obtained are expressed as the reciprocal of the dilution which gives agglutination of 50 per cent of the red cells in the system. These factors and the corresponding degree of agglutination are as follows :

Degree of Agglutination.		Factor.
+++	(Complete) . . .	1·4
++	(Nearly complete) . .	1·2
+	Partial (50 per cent end-point)	1·0
±	(Trace)	0·8

These patterns of the various degrees of agglutination are illustrated in Fig. 1.

FIG. 1.—Diagram showing the pattern of agglutination in varying degrees. Agglutination is complete in the tube at the extreme left and absent in the tube at the extreme right. The pattern shown in the middle tube is that corresponding to the " 50 per cent " end-point.

The use of the factors is shown in the following example :

A tube containing serum in a dilution of 1/800 shows a pattern of agglutination of red cells to a degree corresponding to ++, for which the factor is 1·2. The 50 per cent end-point is given by $800 \times 1{\cdot}2 = 960$, which is taken as the titre of the serum.

For the titration of the serum, tests were set up using different concentrations of polysaccharide for coating the red cells to obtain an idea of the relationship between serum titres and the concentration of antigen used. Three erythrocyte

TABLE I.—*Titration of Standard Serum.*

Cells treated with antigen at concentration of	Serum dilution. 800.	1,600.	3,200.	6,400.	12,800.	25,600.	Saline control.
1/64,000	+++	+++	+	0	0	0	0
1/128,000	+++	+++	+++	+	0	0	0
1/256,000	+++	+++	+++	+++	+	0	0

+++ = Complete agglutination.
+ = Partial (50 per cent) agglutination.
0 = No agglutination.

preparations were made, using antigens in dilutions of 1/64,000, 1/128,000, and 1/256,000, respectively. Unit volumes of these cells were added to 9 volumes of serial two-fold dilutions of antiserum and the tests read after standing for one hour. The schema of the test, and the results, are shown in Table I. It will be observed that, for the range tested, and within the limitations of the method used, the relationship is linear.

STANDARDIZATION OF TEST.

Finally, the sensitivity of the technique was examined under various conditions so that it could be used, as Warburton *et al.* have suggested, to estimate the concentration of polysaccharide present in a solution. For this purpose a polysaccharide preparation was used which had been subjected to repeated careful fractionation precipitation and which, on electrophoresis, appeared to be homogeneous.

Solutions were accurately prepared containing various concentrations of antigen within the range 0·01 to 10·0 μg. per ml. Five volumes of these solutions were added to sets of five volumes of serial two-fold dilutions of standard serum. After allowing the mixtures to stand for 15 minutes, one volume of red cells was added, the cells having been previously sensitized in the concentrations of antigen shown in Table I. The results of some of these titrations are shown in Table II.

In order to reproduce the results graphically so that estimates could be made by interpolation of concentrations of antigen in urine, the degree of inhibition of

TABLE II.—*Standardization of Sensitivity of Test for Detection of Antigen.*

Cells coated with antigen at dilution of	Polysac. conc. μg./c.c.	Serum dilutions. 200.	400.	800.	1,600.	3,200.	6,400.	12,800.	25,600.	Saline control.*
1/64,000	0	+++	+++	+++	+++	+	0	0	0	0
	0·01	+++	+++	+++	+++	+	0	0	0	
	0·1	+++	+++	++	±	0	0	0	0	
	1·0	+++	+	0	0	0	0	0	0	
	10 0	+	0	0	0	0	0	0	0	
1/128,000	0	+++	+++	+++	+++	+++	+	0	0	0
	0·01	+++	+++	+++	+++	+++	+	0	0	
	0·1	+++	+++	+++	±	0	0	0	0	
	1·0	+++	+	0	0	0	0	0	0	
	10·0	0	0	0	0	0	0	0	0	
1/256,000	0	+++	+++	+++	+++	+++	+++	+	0	0
	0·01	+++	+++	+++	+++	+++	+++	+	0	
	0·1	+++	+++	+++	++	0	0	0	0	
	1·0	+++	+	0	0	0	0	0	0	
	10·0	0	0	0	0	0	0	0	0	

+++ = Complete agglutination.
++ = Nearly complete agglutination.
\+ = Partial (50 per cent end point).
± = Trace of agglutination.
0 = No agglutination.

* Control: 10 volumes of saline + 1 volume of sensitized cells.

sensitized red cell agglutination, resulting from neutralization of antibody by antigen in the urine, was expressed as a ratio in the form :

$$\frac{\text{Titre of serum}}{\text{Titre of (serum + polysaccharide)}}$$

For example, from Table II, using cells coated with 1/64,000 antigen, the titre of the serum alone is 3200. The titre of the same serum when mixed previously with polysaccharide at a concentration of 1·0 microgram/ml. is 400. The ratio of inhibition is therefore 3200/400 = 8. When no inhibition occurs, the ratio is 1·0.

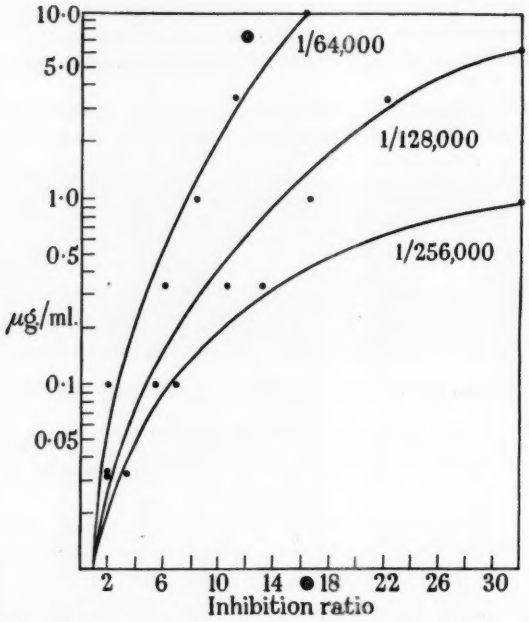

FIG. 2.—Curves showing the amount of inhibition (ordinate) of agglutination produced by the addition of known amounts of polysaccharide (abscissa) to the agglutinating system. Separate curves are shown for red cells sensitized to different degrees.

In Fig. 2 the concentration of polysaccharide is plotted against the inhibition ratio for red cells of the various degrees of sensitization shown in Table II.

To examine the urinary output of antigen in scrub typhus cases the concentration of polysaccharide used for sensitizing red cells was 1/256,000, i.e., four times the end-point concentration. With cells so sensitized the titre of the serum was 12,800.

In performing the tests five volumes of urine, treated as described above, were added to five volumes of two-fold serial dilutions of serum and after mixing, were allowed to stand for 15 minutes. At the end of this period, one volume of sensitized red cells was added, and the tests read after one hour standing. A

TABLE III.—*Excretion of Antigen in Urine According to Day of Disease in μg. per ml.*

Day of disease.	103.	107.	110.	112.	115.	123.	129.	131.	142.	143.	146.	150.	156.	Mean concentration.
1	0·21	..	..	..	..	0·43	..	..	..	..	..	..	..	0·32
2	0·14	..	..	0·08	..	0·11	..	..	..	..	0·11	..	..	0·11
3	0·07	..	..	0·07	..	0·07	..	0·43	..	..	0·21	..	..	0·17
4	0·05	0·11	0·43	0·05	0·04	0·27	..	0·08	..	0·08	0·07	0·08	0·04	0·12
5	0·03	0·07	0·14	0·07	0·05	0·08	0·07	0·07	0·04	0·07	0·08	0·04	0·08	0·07
6	0·03	0·07	0·08	0·03	0·08	0·11	0·07	0·07	0·04	0·02	0·03	0·05	0·07	0·06
7	0·11	0·02	0·07	<0·02	0·08	0·21	0·03	0·03	<0·02	0·02	0·03	0·04	0·02	0·05
8	<0·02	<0·02	0·02	0·02	0·05	<0·02	0·05	0·05	<0·02	0·02	0·04	0·03	0·21	0·04
9	<·002	<0·02	0·14	<0·02	0·08	0·03	..	0·07	0·04	0·07	0·05	0·03	0·08	0·05
10	..	0·03	0·02	0·11	0·07	0·05	0·08	0·05	0·03	<0·02	0·08	0·08	0·07	0·06
11	..	..	<0·02	<0·02	0·21	..	0·14	0·27	<0·02	..	<0·02	0·02	0·14	0·08
12	..	..	0·11	<0·02	<0·02	..	<·02	0·03	<0·02	..	0·03	0·05	<0·02	0·02
:	..	..	..	..	..	..	..	..	..	..	..	..	..	..
19	..	0·03	..	..	0·11	..	..	..	..	..	..	..	..	..
20	..	..	..	..	..	..	..	..	..	..	..	..	..	..
21	..	0·03	..	..	0·11	..	..	..	..	..	..	..	..	..
22	..	..	..	..	0·08	..	..	..	..	..	..	..	..	..
23	..	..	..	..	0·04	..	..	..	..	..	..	..	..	..
24	..	..	..	..	0·03	..	..	..	..	..	..	..	..	..
25	..	..	..	..	0·08	..	..	..	..	..	..	..	..	..
26	..	..	..	..	0·03	..	..	..	..	..	..	..	..	..
27	..	<0·02	..	..	..	..	..	..	..	..	..	..	..	..
28	..	..	..	..	0·05	..	..	..	..	..	..	..	..	..
29	..	..	..	..	0·03	..	..	..	..	..	..	..	..	..
30	..	..	..	..	0·02	..	..	..	..	..	..	..	..	..
31	..	..	..	..	0·02	..	..	..	..	..	..	..	..	..
32	..	..	..	..	<0·02	..	..	..	..	..	..	..	..	..

control titration was done using urine from healthy adult males in place of that being tested; a second control was carried out with saline instead of urine. In addition, each urine tested was controlled by adding to five volumes of it, five volumes of saline and one volume of sensitized cells. Frequent replicate titrations made by each of us separately indicate that results are readily reproducible provided that proper care is observed in making dilutions. The figures obtained should not be regarded, however, as more than reasonable approximations.

An attempt was made to compare the sensitivities of the carmine adsorption method of Smorodintzeff and Fradkina and the method we have used. Various amounts of polysaccharide were added to normal human sera in concentrations

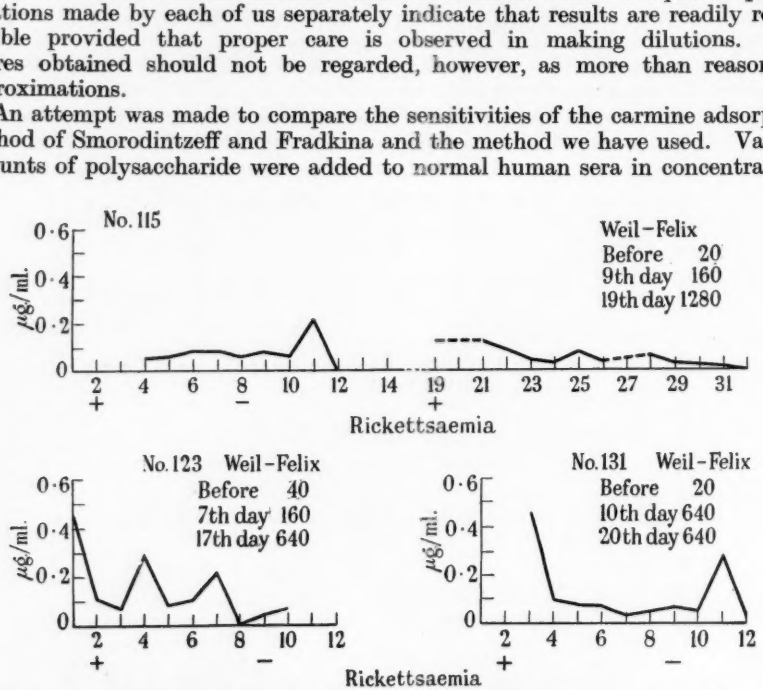

FIG. 3.—Charts from patients No. 115, 123 and 131. The ordinates show the day of disease, the presence or absence of rickettsaemia being indicated by "+" or "—" below the corresponding day. The abscissae show the concentration of antigen in the urine.

ranging from 100 to 0·1 μg. per ml. The carmine test gave positive results with serum containing 10·0 μg. per ml., but not with one-tenth of that concentration.

This method would therefore appear to be much less sensitive than that which we have used, but in attempting to apply the latter as a routine to sera, serious difficulties were encountered with an inhibitor apparently of a lipoid nature in some normal sera. Because of our failure to devise a simple and reliable method of overcoming this, we could not pursue the problem further.

The sensitivity of Léon's precipitin test was also investigated. Apart from the difficulties associated with the precipitin test *per se*, the method adopted in this investigation will detect about 1/20th of the amount of antigen that the precipitin method will show. No comparison was made with the fixation test described by Léon.

Urines from 13 patients suffering from scrub typhus were examined. As a rule, a specimen was examined each day the patient was in hospital. In two

instances (No. 107 and 115) specimens were obtained during a febrile relapse associated with a recrudescence of other clinical evidence of the disease (headache and adenitis). All patients were treated with chloromycetin. Table III shows the results obtained, and Fig. 3 demonstrates in graphic form the same data from some of the cases. Fig. 3 also shows the development of the Weil-Felix reaction and the occurrence of rickettsaemia.

As a control of the specificity of the test, 47 specimens of urine, taken at random from healthy adults who had never been exposed to scrub typhus, all gave completely negative results, no inhibition of agglutination being obtained. An opportunity also occurred to examine 12 specimens of urine from 3 cases of murine typhus from the 10th day onwards. All of these caused no inhibition, but when *Proteus X*19 antigen, instead of that from *Proteus XK*, was used to sensitize the red cells, all specimens were positive.

The investigation shows that rickettsial antigen is excreted in the urine during the course of an attack of scrub typhus. Some findings are worthy of notice.

(*a*) In two cases there was such a considerable concentration of antigen in the urine on the day fever began, as to suggest the presence of antigen before the onset of signs and symptoms. We were unable, however, to investigate this possibility.

(*b*) The output of antigen shows a tendency to cyclic variation in a large proportion of cases, with a high output near the beginning of the illness, followed by a rather rapid decline to a comparatively low level, and subsequently a short-lived increase between the 7th and the 12th days of the disease.

APPLICATION OF TEST.

In this section the data are examined from the point of view of their value in enabling an early diagnosis of scrub typhus to be made in the laboratory. The development of a satisfactory diagnostic test applied early in the disease has become of much greater importance since the reports of the outstanding success of chloromycetin in the treatment of the infection (Smadel, Woodward, Ley, Philip and Traub, 1948).

So far, the only diagnostic tests available have been the Weil-Felix reaction of agglutination of *Proteus OXK* and the complement-fixation test using rickettsial antigen. Both these methods are useful only comparatively late in the disease. As far as is known, neither Léon's nor Smorodintzeff's technique has been applied to scrub typhus. The methods of both these workers have given good results in louse-borne typhus, but, as the comparative sensitivity tests have shown, the technique we have used is capable of demonstrating considerably lesser concentrations of antigen.

In employing the data we have obtained as a diagnostic test suitable for routine and for occasional use by diagnostic laboratories, it was considered wise to fix an arbitrary level of inhibition of agglutination below which any inhibition was disregarded. The purpose of this assumption was to avoid the occurrence of false positive results which might occur where sufficiently accurate techniques cannot be employed, or where unfamiliarity with the method, due to infrequent demand for the test, might lead to errors.

For the purpose of this part of the work, the amount of inhibition occurring has been expressed as the number of tubes by which the 50 per cent end-point has been reduced. The significanct level of inhibition chosen was one tube, equivalent to a serum inhibition ratio of 2. Any urine which gave a degree of inhibition greater than one tube, i.e. a serum inhibition ratio greater than 2, was regarded as being positive, and indicative of infection by *Rickettsia orientalis*.

Fig. 4 shows the amount of inhibition occurring from day to day in the cases observed. The degree of inhibition occurring is shown as the number of tubes "shift" in the end-point. For purposes of correlation with the clinical picture, Fig. 4 includes the duration of fever, the occurrence of rickettsaemia, and the date of chloromycetin therapy. The clinical data used formed part of a study by Smadel and others on chloromycetin in scrub typhus.

In Table IV the same results of the test are shown as "positive" or "negative" after applying the limitation above mentioned of the level of significance of the test.

TABLE IV.—*Occurrence of Positive Results in Relation to Day of Disease.*

Day of disease.	Results in Case No.												
	103.	107.	110.	112.	115.	123.	129.	131.	142.	143.	146.	150.	156.
1	+	..	..	..	..	+(c)	..	..	..	..	..	..	..
2	+(c)	..	..	+	..	+	..	..	..	..	+	..	..
3	+	..	..	+(c)	..	+	..	+	..	..(c)	+	..	..
4	+	+	+	+	+	+	..	+	..	+	+	+	+
5	+	+	+	+	+(c)	+	+	+	+(c)	+	+	+	+
6	+	+(c)	+(c)	+	+	+	+(c)	+(c)	+	—	+	+(c)	+(c)
7	+	—	+	—	+	+	+	+	—	—	+	+	—
8	—	—	—	—	+	—	+	+	—	—	+	+	+
9	—	—	+	—	+	+(c)	..	+	+	+(c)	+	+	+
10	..	+	—	+	+	+	+	+	+	—	+	+	+
11	..	..	—	—(c)	+	..	+	+	—	..	—	—	+
12	..	..	+	—	—	..	—	+	—	..	+	+	—
:	..	..	..	..	..	..	..	..	..	..	..	..	..
19	..	+	..	..	+	..	..	..	..	..	..	..	..
20	..	..	..	..	..	..	..	..	..	..	..	..	..
21	..	+	..	..	+	..	..	..	..	..	..	..	..
22	..	..	..	..	+(c)	..	..	..	..	..	..	..	..
23	..	..	..	..	+	..	..	..	..	..	..	..	..
24	..	..	..	..	+	..	..	..	..	..	..	..	..
25	..	..	..	..	+	..	..	..	..	..	..	..	..
26	..	..	..	..	+	..	..	..	..	..	..	..	..
27	..	—	..	..	..	..	..	..	..	..	..	..	..
28	..	..	..	..	+	..	..	..	..	..	..	..	..
29	..	..	..	..	+	..	..	..	..	..	..	..	..
30	..	..	..	..	—	..	..	..	..	..	..	..	..
31	..	..	..	..	—	..	..	..	..	..	..	..	..
32	..	..	..	..	—	..	..	..	..	..	..	..	..

.. Indicates no test done.
(c) Denotes chloromycetin therapy commenced after urine specimen collected.

It will be seen that in the two cases examined on the day of the onset of fever, the test was positive. Case No. 142 was the only one from which rickettsiae could not be isolated, but the serum showed a rise of Weil-Felix agglutinins against *Proteus OXK* from 20 to 640 on the 10th day of the illness, a sufficient indication of the occurrence of the disease.

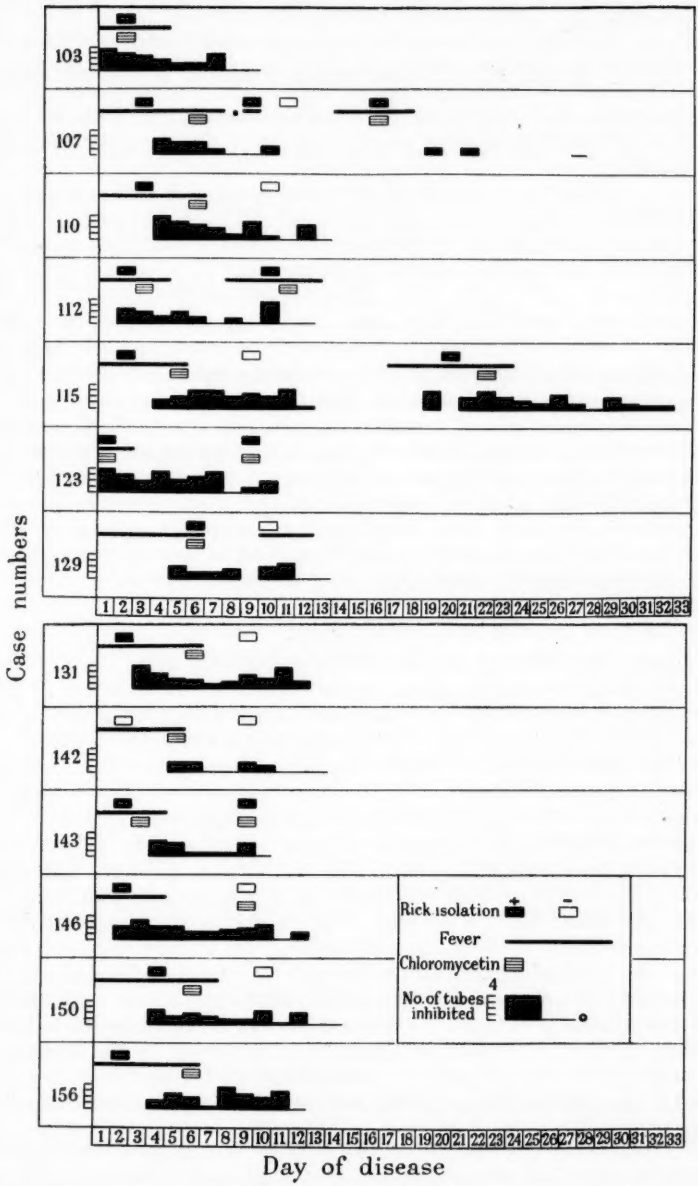

FIG. 4.—Chart showing amount of inhibition of agglutination of sensitized red cells produced by patients' urine, co-ordinated with clinical progress of illness.

Table V summarizes the frequency of positive and negative results according to the day of illness.

TABLE V.—*Frequency of Positive Results According to Day of Disease.*

	Day of disease.											
	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.
Number of cases positive .	2	4	5	11	13	12	8	6	9	10	4	4
Number of cases negative .	0	0	0	0	0	1	5	7	3	2	5	5
Total number of cases . .	2	4	5	11	13	13	13	13	12	12	9	9
Percentage positive .	100	100	100	100	100	93	62	46	75	83	44	44

In addition to the investigation of 13 cases of the evident disease, examinations were made of a few specimens of urine from seven persons who had been exposed to infection. At the time of commencing the observations, these individuals had just completed a course of chloromycetin therapy, extending over a period of four weeks, designed to suppress infection. All these subjects had evidence of rickettsial infection, acquired during the period of exposure, as shown by the development of a positive Weil-Felix reaction, or rickettsaemia, or of typical local eschars or combinations of these. Some had transient fever. In all cases the test gave clear positive results.

DISCUSSION.

The method described of estimating the presence in urine from scrub typhus patients of rickettsial antigen appears to be specific, for no evidence of inhibition of agglutination of sensitized cells has been found with normal urines nor with 12 specmens of urine from 3 cases of murine typhus (another and very similar disease of rickettsial origin) whereas it is additional evidence that, in these latter cases, the substitution of polysaccharide from *Proteus X*19 enabled us to demonstrate the presence of murine rickettsial antigen in the specimens.

The excretion appears to commence at least with the onset of fever, and quite possibly during the incubation period. At this stage, and for the first three days of the fever, the polysaccharide concentration in the urine is at a comparatively high level, and then shows a decline. One of the most interesting features of the rate of excretion is the well-marked tendency for a short-lived but significant increase in the level of concentration about 7 to 10 days after the onset of the clinical disease. No facts are at present available to throw any light on the cause of this increased output. It is possible that it might be related to a particular stage in the process of rickettsial multiplication in the tissues, but at present such a view must be purely speculative.

Neither is any explanation available yet for the continued excretion of antigen long after antibodies to it are demonstrable. The presence of antigen must be taken as evidence of the continued presence of rickettsia in the body. Case No. 115 for example, shows the presence of rickettsaemia with a coincident Weil-Felix titre of 1280. Zinsser's (1934) view of the epidemiology of Brill's disease suggests a similar phenomenon in man for *R. prowazeki*. Fox (1948) reviewed

the literature with regard to the persistence of most of the rickettsiae. His findings are most pertinent to the problem posed here. In mice, for at least two years after recovery from experimental scrub typhus, there was abundant evidence of substantial immunity both on *in vitro* test and when reinfection was attempted. Rickettsiae could always be isolated from the blood, the liver, the brain and the kidney, the last named organ being the most infectious for mice on subsequent passage. It is worthy of note, in passing, that the organism had retained unimpaired its virulence for the species despite the persistence of it in a highly immune host for almost the whole life span of the latter.

For the purpose of using the test as a diagnostic aid, it is recommended that the technique used in the present investigation be adhered to closely for the present, as it is clear that the sensitivity of the method can be altered within wide limits. Under the conditions given, and within the limitations set out concerning the minimum significant degree of inhibition of agglutination, it appears that the detection of rickettsial antigen in the urine by the method described enables the diagnosis of scrub typhus to be made in the laboratory rapidly and in the earliest stages of the disease. The limitation imposed will, it is evident, be liable to give a false negative result on occasion. The data suggest, however, that such results may be uncommon in the first week of the disease at any rate, and that a repetition on the following day will disclose such an event. It must be emphasized, however, that the urine tested be sterile from the beginning. *Proteus XK* has, in the past, been isolated from urine of persons not suffering from scrub typhus, and the presence of it will lead, of course, to false positive results. The few tests that have been made with specimens from cases of murine typhus using OX19 polysaccharide hold out promise of a satisfactory application to that disease. Similar work is in train at present on the diagnosis of Rocky Mountain Spotted Fever, using OX19 and OX2 polysaccharide.

Until further work has been done it is not known whether differentiation can be made between leptospirosis, rat-bite fever, and tsutsugamushi. The occurrence at times of *Proteus OXK* agglutinins to significant titres in leptospirosis is known (Zarafonetis, Ingraham and Berry, 1946), and Savoor and Lewthwaite (1941) showed that rat-bite fever will also produce a Weil-Felix response against *Proteus OXK*. There is as yet no knowledge whether both of these reactions are due to the presence in the causal organisms of Castañeda's (1934) X factor of *Proteus XK*, upon which the present work depends, or upon another antigen common to them and to *Proteus XK*, but not to *R. orientalis*.

SUMMARY.

A method for the estimation of rickettsial antigen in the urine of scrub typhus patients has been described.

The detection of this antigen allows a laboratory diagnosis of the infection to be made in the first day of the disease.

The significance of the excretion of antigen is briefly discussed.

Certain acknowledgements have already been made in the early part of this report. It is also desired to thank Dr. F. G. Morgan, Director, Commonwealth Serum Laboratories, for his interest, and for permitting one of us (J. L. O'C.) to go to Malaya to complete the study. Our thanks are also due to Dr. E. V.

Keogh of these Laboratories, for his helpful interest, and to Dr. J. E. Smadel, who originally suggested the idea, for his help and advice in the preparation of this paper.

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THE ESTIMATION OF PLASMA-SALICYLATE LEVELS.

M. J. H. SMITH AND J. M. TALBOT.

From the Department of Chemical Pathology, King's College Hospital Medical School, Denmark Hill, London.

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SODIUM salicylate is widely used in the treatment of acute rheumatic fever and the more chronic forms of rheumatism. Reid (1948) considers that effective treatment of rheumatic fever with salicylate demands that the dosage should be controlled by repeated determinations of plasma-salicylates, firstly because cure of the disease depends not only on reaching, but on maintaining a high plasma-salicylate level (between 30 and 40 mg. per 100 ml.); secondly, because the variation in plasma levels of patients receiving the same doses of drug is great; and thirdly, because the initial peak is followed by a fall, so that in some patients the plasma salicylate may reach so low a level that a relapse will occur unless the dose of the drug is increased. A minimum level of 35 mg. per 100 ml. was advocated by Coburn (1943), but Graham and Parker (1948) demonstrated that this level, if it occurred within the first seven days of salicylate therapy, appeared to be the point at which the well-known toxic effects of salicylate on the gastro-intestinal tract and on the special senses were noted. These workers stressed the importance of repeated determinations of plasma-salicylate levels, in order that patients with levels of 35 mg. or over, could be observed most carefully for undesirable developments.

Frequent estimations of plasma-salicylate levels demand a rapid and accurate method which requires only a minimum amount of plasma, a consideration of particular importance when the patients are children. Existing photometric methods of analysis are based on the following colour reactions:

(1) The violet colour given with ferric nitrate after extraction with ethylene dichloride (Brodie, Udenfriend and Coburn, 1944).

(2) The red colour given with sodium nitrite and copper sulphate in acetic acid (Mallick and Rehmann, 1945). The same colour reaction was used by Reid (1948) after a preliminary extraction with iso-amyl alcohol at pH 2.

(3) The yellow colour given with sodium hydroxide after previous nitration (Volterra and Jacobs, 1947).

(4) The blue colour given on incubation with sodium carbonate solution after the addition of an alcoholic solution of the Folin-Ciocalteu phenol reagent (Weichselbaum and Shapiro, 1945).

In our opinion, none of these methods is entirely suitable for repeated determinations of plasma-salicylate levels; e.g. the method of Brodie *et al.*, though accurate, involves the use of the toxic ethylene dichloride and requires several mechanical shakings, centrifugations and aspirations, which are tedious and time-consuming. The other procedures are also lengthy, requiring at least an

hour for completion and the majority need 1 ml. or more of plasma for a single determination. The use of a fluorophotometer in the method of Saltzmann (1948) militates against its general employment in hospital laboratories.

We have therefore devised a photometric method of estimation which requires only 0·2 ml. of plasma or serum and can be completed within 15 minutes.

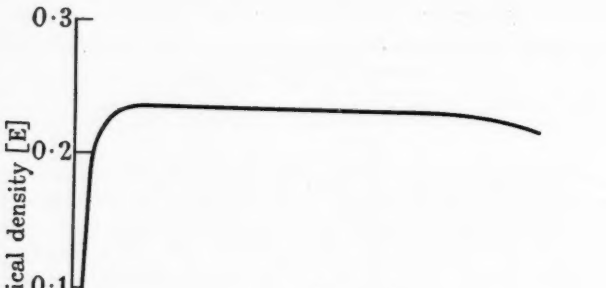

FIG. 1.—Rate of development and fading of Folin-Ciocalteu blue colour at room temperature.

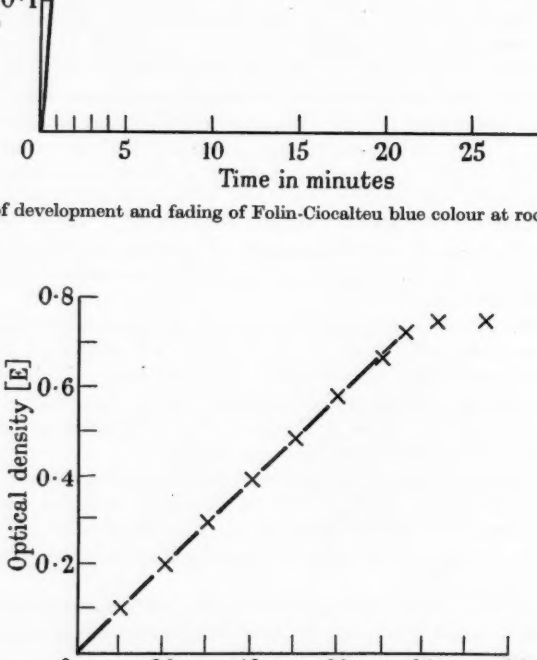

FIG. 2.—Calibration curve of salicylates in distilled water, using the blue colour given by the Folin-Ciocalteu reagent in alkaline solution. The colour intensities were measured in a Hilger Spekker absorptiometer using an Ilford spectrum red filter, No. 608.

EXPERIMENTAL.

The method is based on the reaction of salicylates with an aqueous solution of the Folin-Ciocalteu phenol reagent (Folin and Ciocalteu, 1927). A blue colour having an absorption maximum at 670 mμ develops on the addition of sodium hydroxide; the colour intensity reaches a maximum after 3 minutes, but begins to fade after 20 minutes and is reduced to 90 per cent of the maximum intensity after two hours (Fig. 1). The absorption density of the coloured solution was found to be directly proportional to the concentration of salicylate ion up to 75 mg. per 100 ml. of plasma with the procedure used. The method gave a recovery of 97 per cent from plasma or serum, the blank values of the samples of plasma or serum used were determined and these results were allowed for in the calculation of the recovery rate.

The method of estimation is as follows: 0·2 ml. of plasma or serum is added to 5·8 ml. of distilled water. 3 ml. of Folin-Ciocalteu reagent (diluted 1 to 3 with distilled water) are added and the mixture centrifuged for 5 minutes. 4 ml. of the supernatant liquid are removed and 2 ml. of 1·5 N sodium hydroxide added. The solution is allowed to stand for 3 minutes and the absorption density measured against distilled water in a photo-electric absorptiometer using a 1 cm. cell and a filter transmitting maximally above 670 mμ. We have employed a Hilger Spekker photo-electric absorptiometer and an Ilford spectrum red filter No. 608.

A calibration curve (Fig. 2) may be constructed from dilutions of salicylates in distilled water. Seventy blood samples were obtained from patients not receiving salicylate therapy, and these samples gave a blue colour in excess of the reagent blank; the mean value was equivalent to 7·5 mg. of salicylate per 100 ml. and the standard deviation was 1·5. The extreme values found were 4·5 and 10·5 mg. of salicylates per 100 ml. An alternative calibration curve may be constructed from dilutions of salicylates in citrated plasma from stored whole blood or from pooled sera of patients not receiving salicylate therapy.

RESULTS.

Ten samples of plasma were obtained from patients receiving from 9 to 13 g. of sodium salicylate or calcium aspirinate per day. In addition 20 dilutions of sodium salicylate in normal plasma were prepared. These samples were analysed by the above method, the mean value of 7·5 mg. being used as the correction for the plasma blank, and also by the methods of Brodie *et al.* (1944) and Volterra and Jacobs (1947). The results, expressed as mg. of salicylate per 100 ml. are given in Table I.

DISCUSSION.

The colour reaction with the Folin-Ciocalteu reagent depends on the reduction by phenols of hexavalent molybdenum and hexavalent tungsten to coloured products of lower valence. The method therefore estimates not only salicylate ion, but also metabolic products of salicylic acid such as gentisic and salicyluric acids, which also contain free phenolic groups.

Plasma from patients not receiving salicylate therapy give blue colours in excess of the reagent blank. Substances which react with the reagent in alkaline solution to give a blue colour and which may contribute to the blank value include amino-acids, especially tyrosine and tryptophane, and also uric acid.

The only substance which is commonly encountered at increased levels in pathological sera is the last and we have found that 2 mg. of uric acid are equivalent in colour production to 1 mg. of salicylate. The method of Weichselbaum and Shapiro (1945) gives much higher blank values (13 to 24 mg. per 100 ml.), and we consider that this reduces the accuracy of their procedure.

TABLE I.

Number.	Method of Brodie *et al.*	Method of Volterra and Jacobs.	Present method.
1	28·0	28·5	29·0
2	25·5	25·0	26·0
3	29·0	29·5	30·0
4	11·5	13·0	13·0
5	44·0	41·0	46·0
6	29·0	27·0	28·0
7	42·0	40·0	47·0
8	50·0	48·0	50·5
9	35·0	40·0	38·5
10	54·0	50·0	53·5
11	36·0	31·0	36·0
12	33·5	30·0	33·5
13	40·5	38·0	42·0
14	43·5	38·5	42·0
15	39·0	38·0	38·0
16	42·5	40·5	44·0
17	35·0	34·0	36·5
18	27·5	27·5	30·5
19	25·0	24·5	27·0
20	19·5	17·5	21·5
21	28·0	25·0	29·0
22	23·5	19·5	23·0
23	31·0	31·5	30·0
24	26·0	24·0	25·0
25	28·0	29·0	30·5
26	20·0	20·5	20·0
27	26·0	22·5	27·5
28	30·5	28·5	28·5
29	37·0	36·5	37·0
30	33·5	32·0	33·0

No. 1–10 are actual cases receiving salicylate therapy. No. 20–30 are dilutions of salicylates in normal plasma.

SUMMARY.

A photometric method is described for the estimation of salicylates in plasma or serum, which is based on the blue colour given by salicylates with the Folin-Ciocalteu phenol reagent in alkaline solution.

The method requires only 0·2 ml. of plasma or serum and can be completed within 15 minutes.

Results given by this method are compared with those obtained by two independent procedures for the estimation of salicylates in blood.

We should like to thank Miss C. de Courcy, B.Sc., for able technical assistance and Professor C. H. Gray for advice and criticism. The Spekker photo-electric absorptiometer used in the work was purchased from a grant to Professor Gray from the Central Research Fund of the University of London.

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THE CONCENTRATION OF TRYPTOPHAN, CYSTINE, TYROSINE, PHENYLALANINE, HISTIDINE AND METHIONINE IN NORMAL HUMAN URINE—MICROBIOLOGICAL ASSAY.

S. L. TOMPSETT AND J. FITZPATRICK.

From the Biochemical Department, Royal Infirmary, Glasgow.

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THERE are a number of clinical conditions in which an increased excretion of amino-acids in the urine is known to occur. Quantitative studies have tended to be limited to a restricted number of amino-acids, for which a more or less specific chemical reaction is available. A large amount of published work has been concerned with the increased urinary excretion of histidine that occurs in pregnancy (Kapeller-Adler, 1941) and other publications (Langley, 1940 ; Chattaway, 1947). Chemical methods were used in these studies. Lawrie (1947) has studied the urinary excretions of tyrosine by means of the specific enzyme—*l*(-) tyrosine decarboxylase. This writer noted an increase in excretion during pregnancy and suggested that apart from histidine and tyrosine, other amino-acids may be increased in this condition. More general methods are now available and no doubt their application will result in the accumulation of much needed data. Dent (1946, 1947) has applied the principles of paper chromatography to this subject.

Microbiological methods of assay are now available for the determination of most of the generally known amino-acids. These are similar in principle to those employed for the assay of members of the Vitamin B complex, e.g. riboflavin and nicotinic acid (Fitzpatrick and Tompsett, 1949). These are somewhat more laborious than paper chromatography, but could quite probably be used in conjunction. It is possible by this procedure to determine with reasonable accuracy the quantities of a large number of amino-acids in normal urine (Woodson, Hier, Soloman and Bergeim, 1948 ; Eckhardt and Davidson, 1949 ; Harvey and Horwitt, 1949). Basically the composition of media for the assay of amino-acids is similar to those used for the assay of various members of the Vitamin B complex. The media for the assay of tryptophan can be prepared from casein hydrolysate—this is possible since all tryptophan present in the original casein is destroyed by the process of acid hydrolysis. Until recently it was necessary to prepare media for the assay of other aminoacids from a mixture of amino-acids instead of from peptone or casein hydrolysâte. Lyman, Moseley, Butler, Wood and Hale (1946) have shown that when peptone is treated with hydrogen peroxide cystine, tyrosine, methionine and tryptophan are destroyed, and such may be used as a basis for the assay of these amino-acids. In general only the *l*-enantiomorphs are active for the growth of organisms and hence only these are determined by microbiological assay. There are however some exceptions. Both *d*- and *l*-methionine are available for the growth of *Lactobacillus fermenti* 36 (Barton-

Wright and Curtis, 1948). There is, however, no evidence that the unnatural forms of the amino-acids are excreted in human urine.

In the present investigation, the microbiological assay of tryptophan, cystine, methionine, tyrosine, histidine and phenylalanine in urine has been examined and the concentration of such in normal urine determined.

EXPERIMENTAL.

The following methods with modifications were employed :

Tryptophan (*Barton-Wright*, 1946).—The medium is prepared from casein hydrolysate and the assay is carried out with *L. arabinosus* 17/5.

Cystine (*Barton-Wright*, 1946).—The medium is prepared from a mixture of amino-acids and the assay is carried out with *L. arabinosus* 17/5. Leucine, valine and isoleucine can also be assayed with this medium.

Phenylalanine and histidine (*Barton-Wright*, 1946).—The medium is prepared from a mixture of amino-acids and the assay is carried out with *Leuconostoc mesenteroides* P.60. Methionine and lysine can be assayed with this medium. In the present investigation, owing to contamination of certain of the constituents a methionine free medium suitable for the assay of this amino-acid could not be prepared. Histamine was found to be microbiologically unable to replace histidine.

Cystine, methionine and tyrosine (*Barton-Wright and Curtis*, 1948.)—The medium is prepared from peroxide-treated peptone and the assay is carried out with *Leuconostoc mesenteroides* P.60. Tryptophan can also be assayed with this medium with *L. arabinosus* 17/5 as the organism.

The content of " free " tryptophan, cystine, phenylalanine, tyrosine and methionine in 12 normal urines was determined and the results are shown in Table I. These results were obtained by assaying the urine directly without any previous chemical treatment.

TABLE I.—*The Daily Excretion of Tryptophan, Tyrosine, Cystine, Phenylalanine, Histidine, and Methionine by* 12 *Normal Individuals.*

Amino-acid.	Minimum (mg.).	Maximum (mg.).	Average (mg.).
Tryptophan	9·4	30·2	19·5
Tyrosine	7·5	34·0	18·5
Cystine	104	182	150
Phenylalanine	24	54	38
Histidine	82	403	174
Methionine.	Less than 1 mg. per diem.		

Recoveries of these amino-acids added to normal urine were of the order 85–105 per cent.

The " free " amino-acids so determined would include such in the free state and any present as peptides. The microbiological activity of the latter could be complete, partial or *nil*. Samples of urine were subjected to (*a*) alkaline hydrolysis, (*b*) acid hydrolysis. Tryptophan was determined in samples following alkaline hydrolysis. Tryptophan is said to be stable to alkaline hydrolysis although complete racemisation results. Final results must therefore be doubled to give the correct amount of original *l*-tryptophan. In the present series

alkaline hydrolysis resulted in a 0 to 100 per cent increase in tryptophan content. Acid hydrolysis is assumed not to result in racemisation. When samples of urine which had been subjected to acid hydrolysis were assayed for cystine, phenylalanine and histidine, lower figures were obtained in many cases. It was assumed then that either there was actual destruction or some racemisation as the result of this treatment. Woodson *et al.* (1948) have also noted the destruction of cystine as the result of acid hydrolysis of urine.

The present writers therefore prefer to determine the "free" amino-acid content of urine without resort to alkaline or acid hydrolysis.

The results indicate that normal human urine contains appreciable quantities of tryptophan, tyrosine, phenylalanine and cystine. The figures obtained are in close agreement with those obtained by American workers. Figures for histidine and tyrosine are similar to those obtained by methods other than microbiological (*cf.* Chattaway, 1947); Lawrie, 1947). Methionine was found to be present in much lower concentrations than by American workers.

SUMMARY.

The concentration of tryptophan, cystine, tyrosine, phenylalanine, histidine and methionine in normal human urine has been determined by microbiological methods.

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THE ENHANCEMENT OF INFECTION DURING SHOCK PRODUCED BY BACTERIAL TOXINS AND OTHER AGENTS.

A. A. MILES AND JANET S. F. NIVEN.

National Institute for Medical Research, London, N.W.7.

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In a study of dermal infection in the guinea-pig by staphylococci and clostridia we demonstrated that simultaneously injected adrenaline temporarily diminished the supply of blood, and therefore of phagocytes, to the infective lesion (Evans, Miles and Niven, 1948), and suggested that the associated enhancement of infection was a consequence of this local inhibition of diapedesis. A similar inhibition of diapedesis in lesions induced by tubercle bacilli and staphylococci was observed by Delaunay and Pages (1945) in guinea-pigs severely intoxicated by the endotoxin of *Salm. typhi*. Delaunay, Lebrun and Cotereau (1947) later pointed out the resemblance of the acute intoxication to traumatic shock. These observations suggested to us an exploration in experimental shock of the relation of inhibition of diapedesis and the enhancement of infection, already indicated by our work on adrenaline.

MATERIALS AND METHODS.

Strains of bacteria.

Staph. aureus 21, a strain from Professor Wilson Smith producing no α-toxin, but coagulase-positive with guinea-pig plasma; *Cl. welchii* SR9; *Ps. pyocyanea* Scudder; *Proteus vulgaris* PR3, a wound pathogen; and *Bact. coli* 88. These strains were used by Evans, Miles and Niven (1948) in their tests with adrenaline. The cells from 24-hour cultures were washed once and resuspended in buffered saline or peptone water; 0·1 ml. of various dilutions were used for intradermal injection. Viable counts were made of the suspensions by the method of Miles and Misra (1938).

Shocking agents.

Crude endotoxins from faecal strains of *Bact. coli* and from *Pr. vulgaris* PR1 were made by differential ethanol precipitation of tryptic digests of acetone-extracted bacterial cells (Raistrick and Topley, 1934). The O somatic antigen of *Sh. shigae* was supplied by Dr. W. T. J. Morgan. The α-toxins of *Staph. aureus* and *Cl. welchii* were dried preparations of material precipitated by $(NH_4)_2SO_4$ from toxic culture filtrates, and dissolved as required. The preparations of magnesium adenosine triphosphate (MgATP) were supplied by Professor H. N. Green. For dehydration shock, 60 per cent (w/v) glucose in distilled water, sterilized by steaming, was injected intraperitoneally.

Stock albino guinea-pigs 280 to 500 g. in weight were used, depilated when necessary by a barium sulphide paste 18 to 24 hours before the experiment.

Intradermal injections were made into the dorsal half of the skin in the thoracic and lumbar region. In the tables and figures the lesion diameters are $\sqrt{Dd}$, where D and d are the major and minor diameters, except for very irregular or elongated lesions, of which the area was measured directly and $1{\cdot}13\times$ the square root of this value used. "Inflammation" (I) means marked hyperaemia and induration; and "necrosis" (N) refers to the area of brown, yellow or grossly haemorrhagic skin, destined, as the lesion aged, to become either scabbed or ulcerated. The inflammatory diameters measured include the central necrotic areas.

The methods of estimating arterial pressures and capillary resistance are described in the appropriate sections.

For histological examination, the skin bearing the lesions was excised, tied without stretching to perspex supports, epithelial side upwards, fixed for 2 hours in corrosive sublimate containing 4 per cent acetic acid, removed from the support and tied again for a further 4 hours with the subcutaneous tissue upwards. It was then transferred to corrosive sublimate alone overnight, and after trimming, the specimen was fixed for a further six hours in corrosive sublimate alone.

EXPERIMENTAL.

The Effect of Shock on Staph. aureus *Skin Infection.*

Shock induced by bacterial endotoxins.

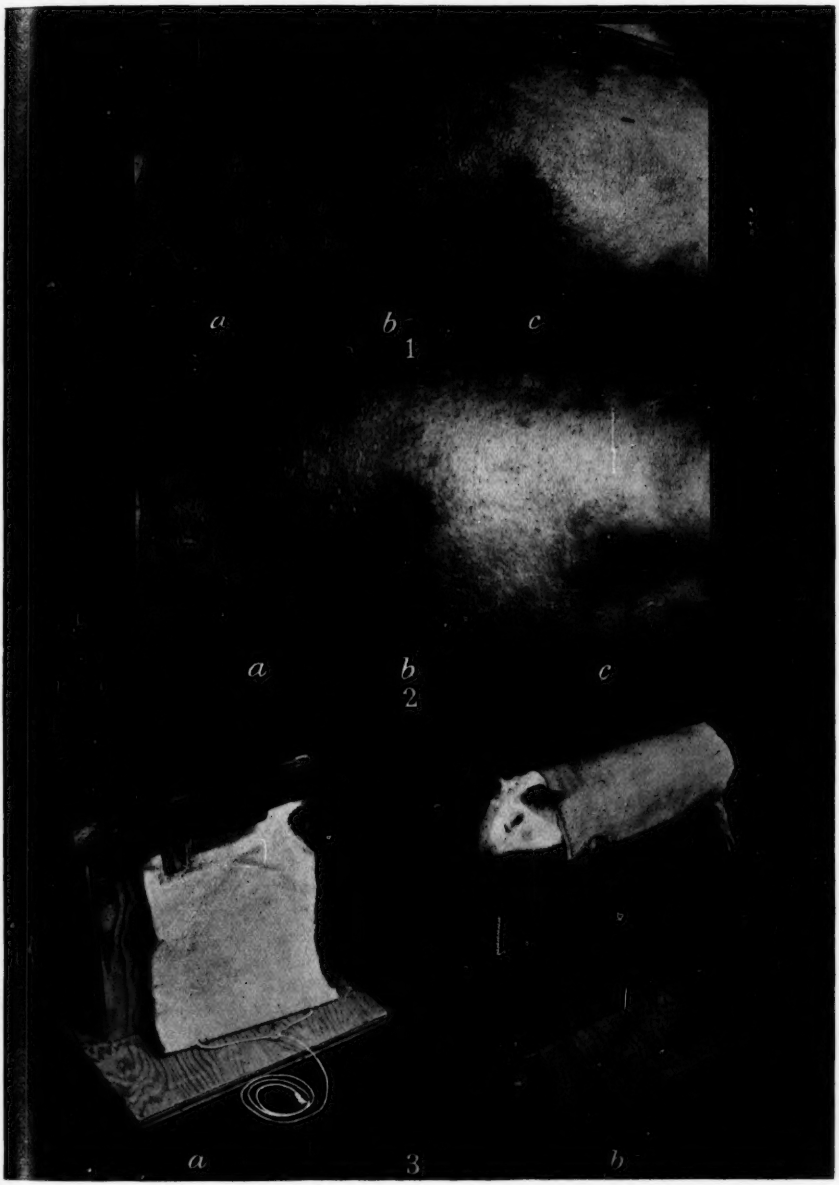
Though *Bact. coli* endotoxin ("colitoxin") was used in most of the experiments, the shock induced and its effect on infection was indistinguishable from that induced by the endotoxins of *Pr. vulgaris* and *Sh. shigae*; and the three may be considered together.

In skin lesions due to dead staphylococci, as Delaunay and Pages (1945) record, the leucocytic reaction was absent. Compared with unshocked animals, the absence was marked in sections of 2-hour-old lesions sampled at the height of shock, and evident in 24-hour-old lesions, when the animals had recovered from a shock lasting 3 to 8 hours. With living staphylococci, 24-hour-old lesions were strikingly different (Fig. 1*c* and 2*c*); for example, in a control animal, they were moderately inflamed and indurated nodules of 5 mm. in diameter, whereas in a shocked animal the same dose of cocci produced a lesion 17 mm. in diameter, with a central area of necrosis of 14 mm. The enhancement depends on the dose of toxin, which in turn determines the severity and duration of shock. In the example in Table I, central necrosis is small and the zone of peripheral inflammation is wide with the smaller doses of toxin; but when the shock lasts 8 to 12 hours, necrosis is extensive and the inflammatory zone is narrow. That is, the longer the shock the smaller the inflammatory reaction during the succeeding hours.

EXPLANATION OF PLATE.

FIG. 1 and 2.—Active and retroactive enhancement of *Staph. aureus* skin lesions by colitoxin shock. Fig. 1, about 5 million living cocci injected; Fig. 2, about 50 million. Photographed after 24 hours. (*a*) No retroaction; lesion 3 hours old when colitoxin was injected. (*b*) Marked retroaction; lesion ½ hour old when colitoxin was injected. (*c*) Direct enhancement; lesion initiated during severe shock, three hours after injection of colitoxin.

FIG. 3.—(*a*) Stand for immobilizing guinea-pigs during blood-pressure determination on the ear. (*b*) Guinea-pig mounted on the stand.



Miles and Niven.

TABLE I.—*The Diameter of 24-hour-old Skin Lesions in Guinea-pigs Injected Intradermally with Graded Doses of* Staph. aureus, *2 hours after Intraperitoneal Injection of* Bact. coli *Endotoxin.*

Dose of living cocci.	Lesion-diameter (mm.). Guinea-pig and dose of toxin in mg.							
	1. 0·00.		2. 0·55.		3. 1·67.		4. 5·00.	
	I.	N.	I.	N.	I.	N.	I.	N.
$4{\cdot}5\times10^6$	11	3	12	6	24	19·5	20	14
$4{\cdot}5\times10^5$	5·5	—	8	3	16·5	14	20	11
$4{\cdot}5\times10^4$	4·5	—	4·5	—	6·5	4·5	8·5	5·5
$4{\cdot}5\times10^3$	3	—	3	—	7	4	5	3·5
$4{\cdot}5\times10^2$	3	—	3	—	5	—	5	—

I = Inflamed area. N = Necrotic area.

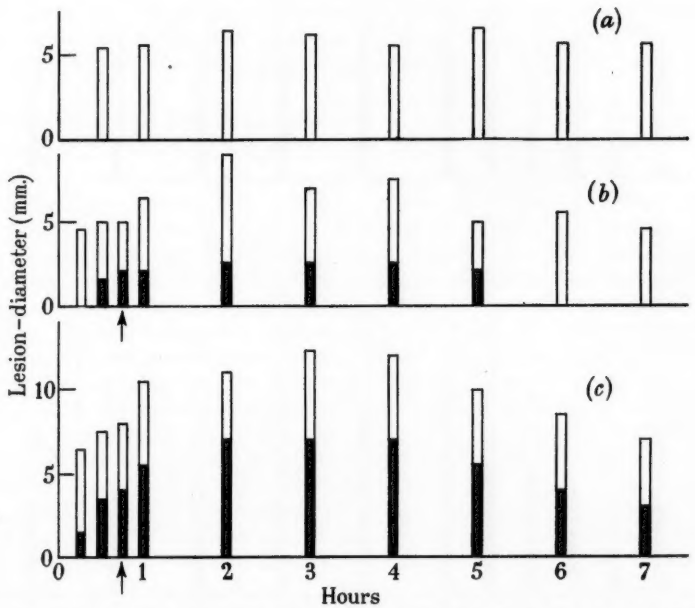


FIG. 4.—The enhancement in three guinea-pigs of lesions due to *Staph. aureus* injected before and during intoxication by intraperitoneal colitoxin. The height of the white and black columns indicate respectively the diameters of the inflamed and necrotic areas. The arrows indicate the injection of toxin. (*a*) No toxin ; (*b*) 1·25 mg. toxin ; (*c*) 5·00 mg. toxin.

As in all the experiments with *Staph. aureus* 21, about 5×10^6 living cocci were injected, being the dose that in the unshocked animal produced an indurated, inflamed lesion 4 to 7 mm. in diameter without central necrosis (Fig. 1*a*).

The time relations of shock and enhancement are exemplified in Fig. 4 and 5, which record the results of typical experiments. Cocci were injected at intervals of an hour or less, and the colitoxin given intraperitoneally (Fig. 4) after the first

30 minutes; the lesions in unshocked animals have the same diameter and severity, proving that the suspension of cocci, held at 2° C. in 0·1 per cent peptone water, retained its infectivity throughout. With 1·25 mg. of the colitoxin, the

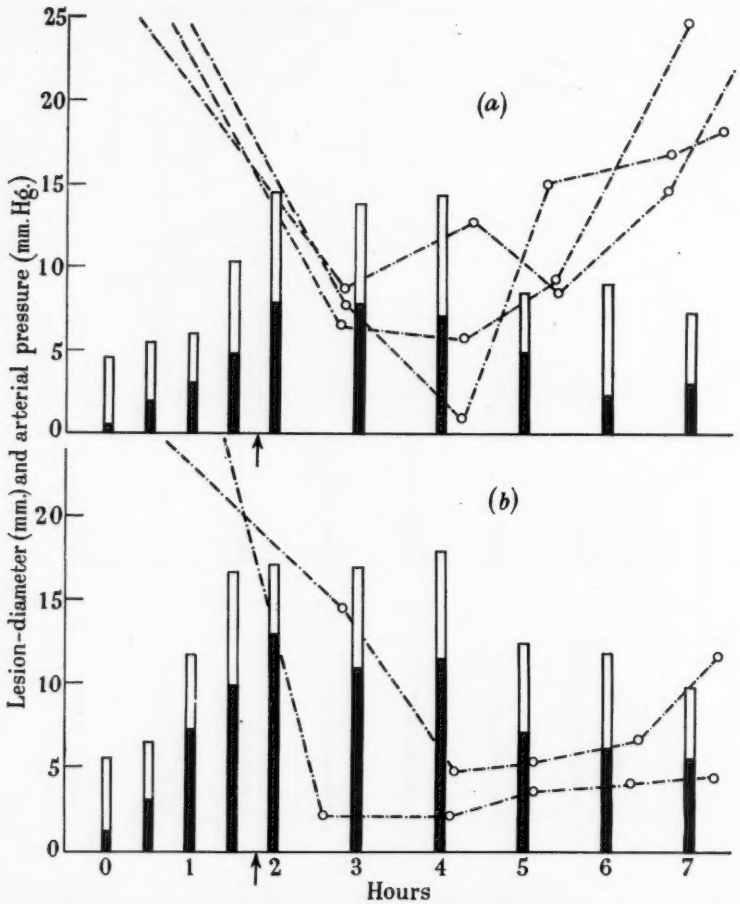


FIG. 5.—The enhancement of lesions due to *Staph. aureus* injected before and during intoxication by intravenous colitoxin. The height of the white and black columns indicate respectively the diameters of the inflamed and necrotic areas. The arrows indicate the injection of toxin. (*a*) 5 mg. toxin. The diameters are means from three guinea-pigs. The arterial blood pressures are recorded separately. (*b*) 7 mg. toxin. The diameters are means from two guinea-pigs. The arterial blood pressures are recorded separately.

4 to 5 hours' shock and enhancement were coincident and cocci injected 2 hours after the animals' recovery were not affected; with 5 mg. enhancement was greater and again all lesions injected during the 7 hours of shock were enhanced.

Retroactivity of Endotoxin Shock.

One feature of Fig. 4 demands special consideration—namely, the enhancement of lesions produced by cocci injected *before* the toxin. The smaller dose of toxin enhanced lesions 15 minutes old, the larger dose lesions that were 30 minutes old at the time of its injection. If shock is responsible for enhancement, it is in this sense "retroactive." With intraperitoneal colitoxin, shock took from 1 to 1½ hours to develop fully. By giving large doses of toxin intravenously (Fig. 5), severe shock was induced within 20 to 30 minutes, and in these instances, staphylococcal lesions already two hours old were enhanced (Fig. 5). In general, the older the lesion and the less severe the shock, the less the retroactitivy. But in none of 16 tests of various shocking agents was it possible to increase the retroactivity of the shock on *Staph. aureus* in the doses used, by more than two hours.

Allowing one hour for the maximum development of the shock, it is clear that in three hours the staphylococcal lesion is fully determined (Fig. 1 and 2); and after this lapse of time the induction of the profoundest shock short of death did not in our hands produce lesions that differed from those in control animals.

The histology of lesions modified by shock.

The progress of the staphylococcal skin lesions was determined by injecting a fixed dose of cocci at intervals, and excising all the injection sites after a given interval; the same animal thus yielded a series of lesions, e.g. 5, 3, 2, 1, ½ and 0 hours old. Shock was induced by colitoxin or hypertonic glucose, in some animals 5 hours, in some 3 hours, and in some 1 hour before the animal was killed and the lesions were excised. Fig. 6 summarizes the characteristic modifications in the tissue leucocytosis and fibrinous exudation in an experiment on four guinea-pigs. In the unshocked animal (Series 1) there was, with increasing age of lesion, a rapid and progressive increase in leucocytosis and exudation, without any noticeable increase in the number of stainable cocci originally injected. Series 2, 3 and 4 illustrate both the action and the retroaction of shock. In Series 2, the leucocytosis was diminished in the 3-hour lesion subjected to shock during the last hour, and suppressed in the ½-hour lesion that was initiated during shock. In Series 3 there was great diminution in the 5-hour lesion subjected to shock during its last 3 hours, and suppression in 3-, 1- and ½-hour lesions made during shock. The picture is similar in Series 4, except that the shock was passing off during the fifth hour, and some leucocytosis had occurred in the ½-hour lesion. In Series 2 and 3, and in the older lesions of Series 4, the number of cocci and the degree of tissue damage increased with the age of the lesion. There was also control necrosis, and at its periphery the capillaries were dilated, and the arterioles contracted, in the 3- and 5-hour lesions of Series 3 and 4.

The abundance of the tissue leucocytosis, therefore, is inversely related to the degree of enhancement exhibited by these lesions when they are allowed to mature for 24 hours in the living animal; and retroactivity in enhancement of infection is paralleled by retroactive diminution of tissue leucocytosis.

The vertical black columns in Fig. 6 represent the number of *healthy* phagocytes, but in shocked animals at least they do not represent the whole leucocytic picture. In all lesions that had been allowed to develop normally before the injection of the shocking agent there were numbers of degenerate polynuclear cells, the

degeneration ranging from mild nuclear and cytoplasmic changes, through pyknotic cells, to masses of karyolytic remnants of nuclei in the tissue spaces of the lesion. The longer the subsequent shock, the greater the degeneration of the phagocytes that had migrated into the tissues before the shock. Either the shock was in some way responsible for the acute degeneration of these leucocytes, or the abundance of healthy polynuclear cells seen in sections of the corresponding lesions in unshocked animals represents an equilibrium state in a continuous invasion of the tissues by blood leucocytes, which replace those whose

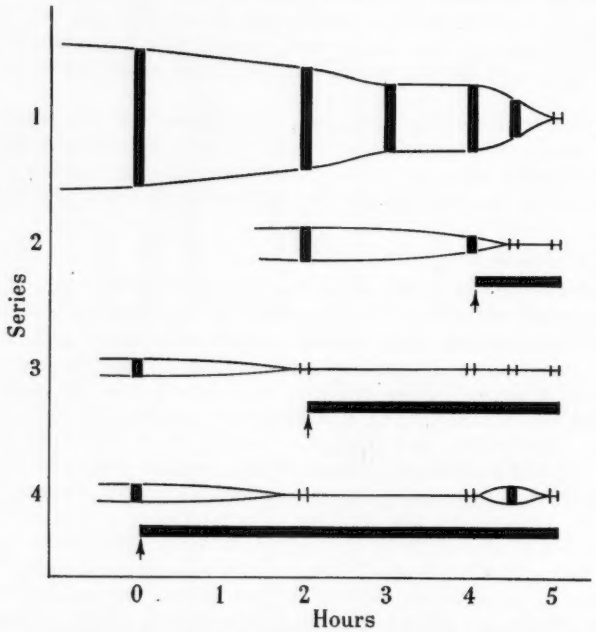


FIG. 6.—Tissue leucocytosis in response to intradermal *Staph. aureus* in normal and shocked guinea-pigs. The heavy vertical lines symbolize the relative amount of healthy phagocytes and fibrinous exudate in lesions initiated at the indicated hours and excised at the fifth hour. The arrow indicates the injection of the shocking agent, and the heavy horizontal lines the duration of the shock.

rapid destruction is a normal feature of the tissue reaction in the early stages of infection. The point is of considerable importance in determining the mechanism of enhancement of infection by shock (see discussion).

Shock induced by bacterial exotoxins.

The bacterial endotoxins induced prolonged shock without killing the guinea-pigs. It was more difficult to demonstrate great enhancement by exotoxins because shocking doses were close to lethal doses, and shock took longer to develop. Nevertheless, the enhancement of skin lesions, its time relation to the period of shock, and its rough proportionality to the severity of shock, were in

essence the same with the preparations of crude α-toxins of *Cl. welchii* and *Staph. aureus* given intraperitoneally. The possibility of a specific aggression action of the staphylococcal toxin on the *Staph. aureus* infection cannot be excluded, but at least it was not due to the α-toxin in the preparation used, since Strain 12 did not produce α-toxin.

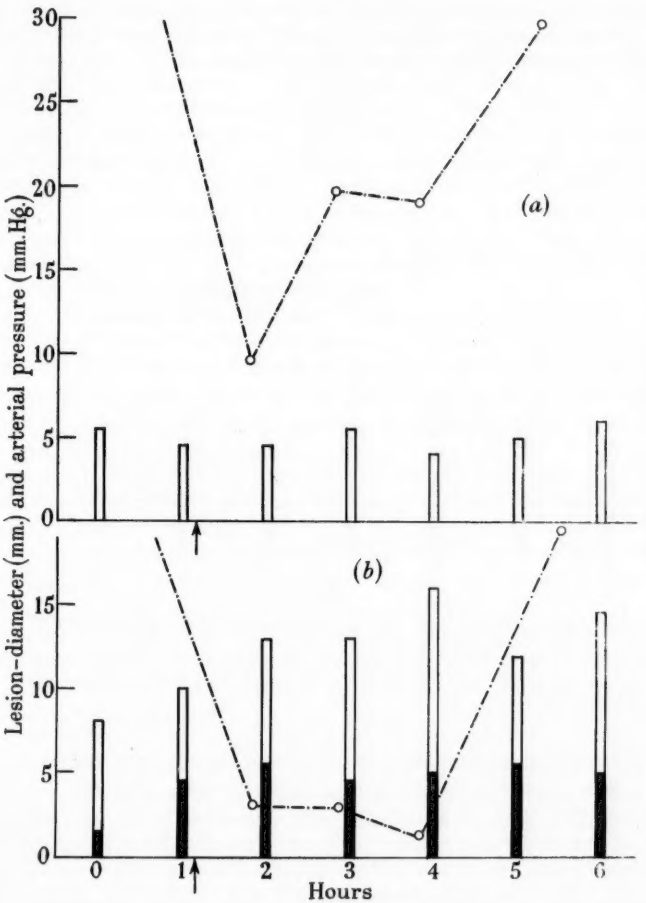


FIG. 7.—The enhancement of lesions due to *Staph. aureus* injected before and during intoxication by intraperitoneal magnesium adenosine triphosphate. The height of the white and black columns indicate respectively the diameter of inflamed and necrotic areas. The arrows indicate the injection of ATP. (*a*) Guinea-pig given 420 mg. ATP/kg. Arterial pressure does not drop below critical level; no enhancement. (*b*) Guinea-pig given 320 mg. ATP/kg. Arterial pressure reaches and remains for 3 hours below critical level; enhancement.

Shock induced by adenosine triphosphate and hypertonic glucose.

Severe but non-fatal shock was produced by 400 to 550 mg. of Professor Green's preparation of MgATP per kg. body weight, and dehydration shock lasting 2 to 6 hours by 15 to 20 ml. 60 per cent glucose per kg. body weight, though 20 ml. per kg. usually proved to be about LD50 for the stock guinea-pigs used. Both substances were given intraperitoneally. Here again (Fig. 7 and 8) the association of severity of shock, and enhancement of infection and degree of retroactivity, was essentially the same as with endotoxin shock.

None of the shocking substances tested modified the coccal lesions unless they were given in doses that induced recognizable shock.

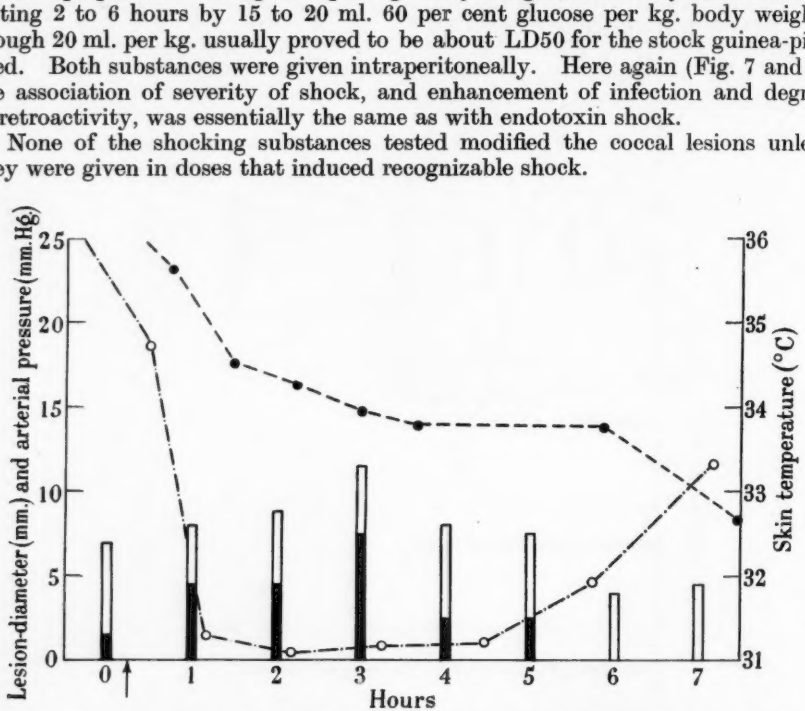


FIG. 8.—The enhancement of lesions due to *Staph. aureus* injected before and during intoxication by intraperitoneal hypertonic glucose solution. The height of the white and black columns respectively indicate the diameters of inflamed and necrotic areas; the open circles, arterial pressures; and the solid circles, skin temperature. The arrow indicates the injection of the glucose.

The Mechanism of Enhancement of Staph. aureus *Infections by Shock.*

After one to two hours' shock, the most striking departure from the norm of inflammation in lesions that later will prove to have been enhanced is the obvious absence of signs of inflammation in the infected skin, and histologically, the absence of phagocytes and a diminution of fluid exudate in the tissues. By analogy with the adrenaline ischaemia (Evans, Miles and Niven, 1948), it is possible that in shock also a local diminution in blood flow is responsible for the absence of leucocytes, and consequently for the relatively unimpeded growth and spread of staphylococci. Pickrell (1938) found that the enhancement of pneumococcal skin infections in the rabbit by ethanol intoxication was associated with absence of local leucocytic immigration, but that it was not due to a depression of phagocytic activity. He suggested that there were no leucocytes in the

infected tissues because the capillaries failed to respond to the injected pneumococci by dilation and increase in permeability.

Delaunay and his colleagues have worked extensively at this problem. Besides inhibition of diapedesis, they found in endotoxin shock that the carotid blood pressure was maintained at a high level until just before the death of the animal (Boquet, Delaunay, Lehoult and Lebrun, 1947). However, in the first 30 minutes of intoxication the skin of the animal became cold and pale, and scarcely bled on cutting (Boquet, 1942), peripheral circulation was diminished and 30 to 40 per cent of leucocytes disappeared from the peripheral circulation (Boquet, Delaunay, Lebrun and Lehoult, 1947*a*). At the same time, the circulation in the vascular beds of both skin and mesentery at first accelerated ; then arteries and to some extent veins underwent transient segmental contraction, followed by dilatation, when the blood flowed more slowly (Delaunay, Lebrun and Delaunay, 1947). The contractions and dilatations alternated until, just before death of a fully shocked animal, dilatation supervened. In the mouse and guinea-pig mesentery the circulation did not cease until just before death. The sequence was similar in the rabbit's ear (Delaunay, Boquet, Lebrun, Lehoult and Delaunay, 1948), but although temporary reversal of flow was observed in some capillaries and venules, there was never a true stagnation of the blood.

The term " inhibition of diapedesis " is question-begging if the meaning of " diapedesis " is properly confined to actual passage of leucocytes through the capillary walls. The absence of tissue leucocytes may be due to changes in the blood, the capillaries or the tissues, or to combinations of such changes, viz. :

(*a*) There may be so little blood flowing as to deprive the capillaries of the leucocytes necessary for diapedesis ;

(*b*) the blood, though flowing, may contain too few leucocytes ;

(*c*) a certain intracapillary pressure necessary to force the leucocytes through the endothelium may be lacking ;

(*d*) the leucocytes may lose an intrinsic power of diapedesis ;

(*e*) the capillaries may become more resistant to the passage in one direction of leucocytes, or of chemiotactic substances in the other ; and finally

(*f*) the infected tissues may not elaborate chemiotactic substances.

Of these possibilities (*b*) seems to us to have been excluded by Boquet, Delaunay, Lebrun and Lehoult (1947*a*) because in the leucopenia of shock the number of circulating leucocytes, though reduced, was still several thousand per mm^3 ; (*d*) is not proven ; and (*f*) is unlikely. With regard to (*a*) and (*c*) there are obviously profound vascular disturbances, but their connection with inhibition of diapedesis is obscure. With regard to (*e*), Delaunay, Pages and Cotereau (1946) describe a great increase in capillary resistance in guinea-pigs treated with shocking and non-shocking doses of endotoxin. Our results are consistent with (*a*) and (*c*)—a combination of diminished blood flow and pressure in the peripheral vessels. We have found no good evidence (p. 86) for an increase in capillary resistance or indeed for the absence of the increase in capillary permeability that characterizes acute infective inflammation.

Peripheral Blood Pressure in Shock.

There is no need to establish contemporaneity of systemic hypotension and enhancement of infection in order to connect the two phenomena causally. As

we have established, shock is retroactive, so that a late systemic hypotension might determine earlier enhancement. Moreover, the carotid blood pressure in shock might be maintained longer at physiological levels than arterial pressures in the skin and other peripheral organs. The passage of blood cells through the true non-muscular capillaries is intermittent (Krogh, 1929; see also Chambers and Zweifach, 1946), and in the resting state of, e.g. the skin, the capillary blood flow is largely shunted directly from the smallest arterioles to the smallest venules through certain channels (the a–v channels of Chambers and Zweifach, 1940, 1944). In progressive shock induced by bleeding or trauma, after an initial reactive period the capillary flow decreases further; and in severe shock, it may cease in the skin, though it still continues in the mesentery (Chambers, Zweifach and Lowenstein, 1943; Zweifach, Chambers and Lowenstein, 1944).

TABLE II.—*The Diameter of 24-hour-old Skin Lesions in the Ear of Guinea-pigs not Shocked (Group A) and Shocked (Group B) with Colitoxin.*

Group.	Dose of living cocci.	Shock.	Lesion-diameters (mm.) in four guinea-pigs. I.				N.			
A	25×10^6	+	8·5,	10,	12,	9	4,	3,	5,	5
B		—	7,	4,	4,	4	2,	1·5,	2,	1·5
A	25×10^5	+	7,	6,	5,	6	3,	2·5,	1·5,	1·5
B		—	3,	1·5,	1·0,	3	0,	0,	0,	1·5

I = Inflamed area. N = Necrotic area.

We measured peripheral blood pressures in the ear of the guinea-pig, thus avoiding operative or anaesthetic procedures that might modify the vascular state of the animal, and were able to make repeated observations on vessels which represented, more closely than the central arteries, the vessels of the skin where enhancement was taking place. The degree of correspondence between ear and skin is hard to estimate. The ear certainly has a greater vascularity, both blood and lymphatic, than the skin of the trunk, and larger doses of living staphylococci are needed to induce good experimental lesions. Nevertheless, both colitoxin and hypertonic glucose will enhance staphylococcal lesions in the ear. In a typical experiment, approximately 25 and 2·5 million living cocci were injected in 0·025 ml. volume into the dorsal skin of the right and left ears respectively of 8 guinea-pigs, about 8 mm. proximal to the midpoint of the lateral margin. Four (Group A) were severely shocked with glucose; four (Group B) were controls (Table II). In shocked animals, the smaller dose of cocci produced mean lesion-diameters of 6·1 mm. inflammation and 2·1 mm. necrosis; the corresponding figures in the controls were 1·4 and 0·4. For the purpose of the tests, then, the ear vessels may be considered representative of those in the skin.

Arterial pressures in the guinea-pig's ear.

Pressures were measured by a modification of the capsule of Grant and Rothschild (1934), consisting (Fig. 9) of capsule, an illuminating device and a ×4 lens of 30 mm. working distance for the observation of small vessels. The guinea-pigs were placed on a narrow wooden platform, grooved longitudinally to take the

body and the chin and notched along the two edges to allow the legs to depend (Fig. 3*a*), and held by a broad linen band, leaving the ears and snout free (Fig. 3*b*). After a few minutes' experience, most animals were quiet enough for repeated observations and their very occasional squirming did not raise the blood pressure significantly. The median arterial tree in the guinea-pig's ear usually branches fan-wise from near the base towards the highly vascularized area round the main marginal veins. One of the smaller branches, 0·08 to 0·12 mm. in diameter, with no offshoots for a length of 2–3 mm. was used for an estimation of the diastolic pressure of the contained blood.

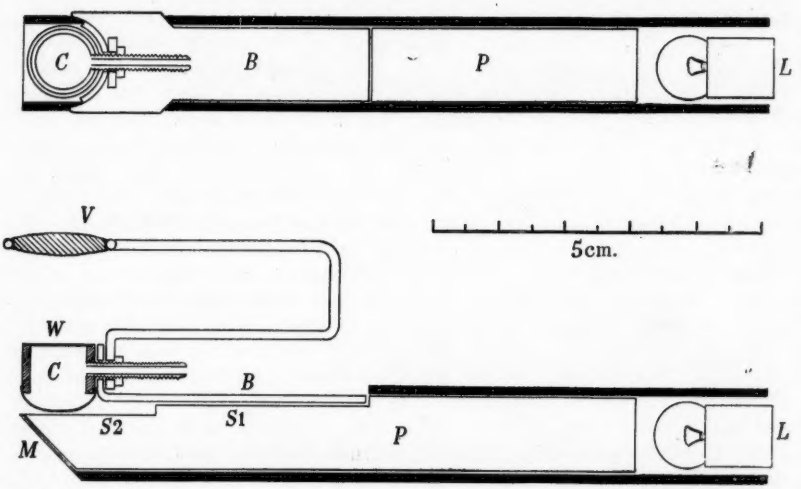


FIG. 9.—Modification of Grant and Rothschild's capsule, for measuring arterial pressure in the ear of guinea-pigs. C, capsule with diaphragm of gold-beater's skin, closed by a glass window, W. L, lamp (2 amp., 2 volt). M, mirror. P, Perspex total-reflection rod in bakelite sheath with a plane surfaces S_1 for the metal base (B) of the capsule-holder, and S_2, for supporting the ear during compression. V, viewing lens (×4) on adjustable soft wire support.

The animals were kept at laboratory temperature, and for each reading, vasodilatation of the arteries was induced by alternate compression (at 100 mm. Hg) and release, 5 to 6 times. In all but severely shocked animals, dilatation could be induced in this way, though the transient segmental contractions of the artery during the earlier stages of shock, noted by Delaunay *et al.* (1948), sometimes did not disappear until after more prolonged stimulation. The values for consecutive readings on vessels in this reactive state were reasonably steady—36, 38, 40, 40, 40, 38, and 6, 4, 8, 8, 6 mm. Hg are typical series at high and low pressures—and 4 to 5 readings were taken and averaged.

The pressures varied to some extent with the diameter of the vessel, and its distance from the base of the ear. But since only comparative readings of arterial pressures in skin were required, and not an indication of the systemic pressure, the variation could be ignored provided that the same piece of artery was used for each determination. The arterial pressure in normal animals lay

between 25 and 50 mm. Hg and in shock declined to <10 mm., 30 to 60 minutes after injection of the shocking agent. In severe shock the pressures were between 1 and 5 mm. Hg ; at these lowest levels the exact values were difficult to determine, even with a water manometer, owing to the insensitivity of the capsule ; but in these circumstances the pressures in the nearby veins of about twice the diameter, judging by their almost simultaneous filling as the capsule pressure was allowed to decline, were only a fraction of a millimetre Hg less than the arterial pressures.

Arterial pressures in inflamed ears.

Inflammation did not materially alter the arterial pressures in shocked animals, but in unshocked animals acute vasodilation of the artery under observation sometimes resulted in higher pressures. The arterial pattern in the middle of both ears of nine guinea-pigs was mapped, and arteries chosen, one in each ear, which gave similar pressure readings. Three animals were injected with an approximate LD50 of hypertonic glucose, and three with colitoxin. Half an hour later all received in one ear 0·03 ml. of a suspension of living *Staph. aureus* sufficient to give 7–10 mm. inflammatory induration in 24 hours. The suspension was injected intradermally over the test arteries and formed a bleb about 7–8 mm. in diameter. About 3 hours later, when all the intoxicated animals were acutely shocked and inflammation was obvious in the control ears, the arterial pressures were again measured (Table III). Only in two of the unshocked animals was there any marked excess of pressure on the inflamed side. In both control and test animals the vascular picture was greatly obscured in the inflamed area, only the larger vessels being visible, and these hazily ; and in the shocked animals the arterio-venous pressure differences were apparently less in the inflamed ear than in the opposite untreated ear. We may conclude that the behaviour of vessels in the untreated ear of shocked animals reflects that of skin vessels in areas infected by the staphylococci.

TABLE III.—*Arterial Blood Pressures Measured in Inflamed and Untreated Ears of Shocked and Normal Guinea-pigs.*

Guinea-pig.	Injection. For shock.	Injection. Into ear.	Arterial pressure (mm. Hg) in ear. Before the experiment.	Arterial pressure (mm. Hg) in ear. 3 hours after the injections.
1	Glucose	Staph.	38	?5
		Nil	42	6
2	,,	Staph.	42	?4
		Nil	40	4
3	,,	Staph.	44	20
		Nil	38	18
4	Colitoxin	Staph.	36	<6
		Nil	36	4
5	,,	Staph.	40	?4
		Nil	42	5
6	,,	Staph.	45	4
		Nil	40	5
7	*Nil*	Staph.	32	28
		Nil	28	28
8	,,	Staph.	36	46
		Nil	42	28
9	,,	Staph.	40	50
		Nil	40	34

The blood pressure in skin arteries during shock.

Fig. 5, 7 and 8 clearly show the association of enhancement and the levels to which arterial pressures drop in the ear. The greater and the more prolonged the drop, the greater both retroactive and direct enhancement (Fig. 5*a* and *b*). Susceptibility to ATP shock varied greatly in the guinea-pigs, and whereas a large dose that failed to lower the arterial pressure below 10 mm. Hg did not enhance infection (Fig. 7*a*), in another animal enhancement occurred with a smaller dose that lowered the pressure below this level (Fig. 7*b*). In none of the shocked animals was there any marked vasodilatation and this, in conjunction with the approximation of arterial and venous pressures observed in all severely shocked animals, suggests a greatly diminished flow of blood in the skin. The chilling of the skin in shock is consistent with this view. The cold skin, however, was not necessarily due to poor circulation. Temperatures were measured by

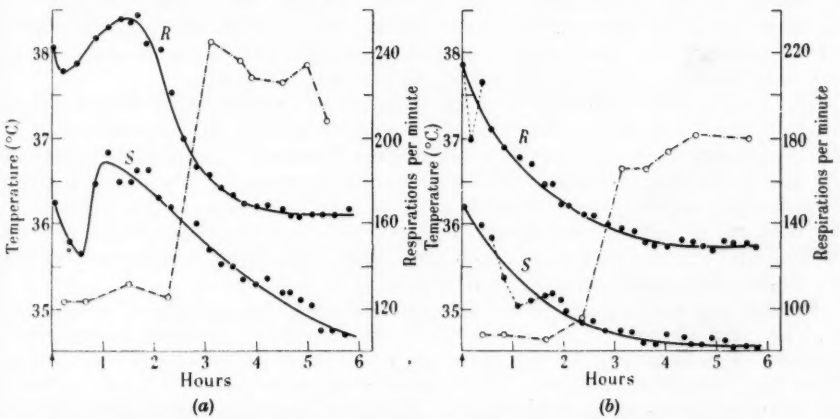


FIG. 10.—The rectal (R) and skin (S) temperatures (solid lines) and respirations (dotted lines) of guinea-pigs during shock. (*a*) Shock due to intraperitoneal colitoxin. (*b*) Shock due to intraperitoneal hypertonic glucose solution.

thermocouples applied to the skin of the thorax under two thicknesses of adhesive tape, or introduced into the gut, 4 cm. from the anus. In colitoxin and dehydration shock, both the skin and rectal temperatures were lowered; and, except for an occasional transient hyperthermia with endotoxin preparations (Fig. 10*a*), a temporary drop in rectal temperatures after intraperitoneal glucose (Fig. 10*b*) and some fluctuations in the skin temperatures during the first hour, the time-temperature curves ran roughly parallel. This sequence was observed in both fatal and non-fatal shock. The cold skin therefore, may result from a good circulation of cold blood, and not a poor circulation of relatively warm blood.

The respiration rate increased when shock became severe (Fig. 10*a* and *b*). Its rapid increase was a useful index of the onset of shock.

In all our experiments with infection and shock, there was no enhancement unless, at some stage during intoxication, the arterial pressures in the ear dropped below 5–8 mm. and the arterio-venous pressure difference became small (*cf.*

Fig. 7*a* and 7*b*). Whether the absence of tissue leucocytes is directly due to a low capillary pressure, or to diminished blood flow, or both, must await microscopic studies *in vivo*, if possible of tissues in their natural state in unanaesthetized animals. Nevertheless, we feel that, with one exception, the facts elicited by Delaunay and his colleagues, and by ourselves, are compatible with the view that changes in the flow and pressure of the capillary blood are sufficient to account for the inhibition of tissue leucocytosis observed in shock.

The Demonstration of Capillary Resistance in the Guinea-pig.

The exception is the increase in capillary resistance after the injection of endotoxin reported by Delaunay, Pages and Cotereau (1946), who found that half an hour after shocking and subshocking doses of *Sa'm. typhi* endotoxin, the minimal suction of one minute's duration required to rupture the capillaries in the dorsal thoraco-lumbar skin had risen from 100–200 mm. to 600–650 mm. Hg. We have confirmed these figures in colitoxin, dehydration and MgATP shock. But we contend that in shocked animals the threshold pressures for rupture can vary independently of the resistance of the capillary walls.

Fig. 11*a* represents the hydrodynamic relation of blood vessels during cupping. A, a, c, v and V are respectively arteries, arterioles, capillaries, venules and veins, with manometers registering the appropriate pressures. The capillary pressure is p, and R the pressure under which the capillaries rupture. The *cuffing* method of measuring capillary resistance depends on the occlusion of the veins only. The pressure in c rises, and when R is reached, petechiae are produced; i.e. given similar arterial pressures, differences in the time taken to produce rupture will reflect differences in capillary resistance.

Ideally, the *cupping* method should be equivalent to applying a suction cup (C_1, Fig. 11*a*) to the top of the capillary manometer; i.e. there is never any occlusion of the vessels. In reality, a cup (C2) is placed over a wide area of skin. When the suction is less than local arterial pressures, veins only are occluded and the effect is the same as in cuffing. With suction pressures of 100–200 mm. Hg, all the vessels at the edge of the cup are occluded and the vascular network within becomes a closed bag of blood, which is ruptured when the atmospheric pressure transmitted through the tissues presses it against the tense dome of skin inside the cup (Fig. 11*b*). The amount of blood within the closed system will depend on the state of the vessels when the cup is applied. As the suction pressure rises, blood will be sucked in after the occlusion of the veins and before the occlusion of the ovaries. If it could be assumed that the vessels within the cup always filled to the same extent, then the pressure required to rupture the distended capillaries would be a function of the resistance of the capillary walls. On the other hand, if there were little blood in the skin and if the local arterial pressures were low, the vessels within the cup would be less distended and a much greater pressure required for their rupture (Fig. 11*c*); and an increased rupture-pressure would result solely from a decreased blood supply; as, e.g., in an ischaemic skin.

The correctness of this interpretation is borne out by the following observations.

Histology of the cupped areas of skin.—A standard suction of 250 mm. Hg was applied for 1 minute to the skin of normal and shocked guinea-pigs, using

a small cup of 11 mm. external, 7 mm. internal, diameter, and 9 mm. deep. The tested areas were immediately excised. In stained sections of sites bearing petechiae, the arterioles were almost empty, the venules partly empty, and the capillaries distended with blood; rupture had occurred mostly at the venous ends of the capillaries. In sites without macroscopic petechiae there was little blood in either arterioles or venules, the capillaries were less distended with blood, and only a few small haemorrhages were seen in the deeper parts of the

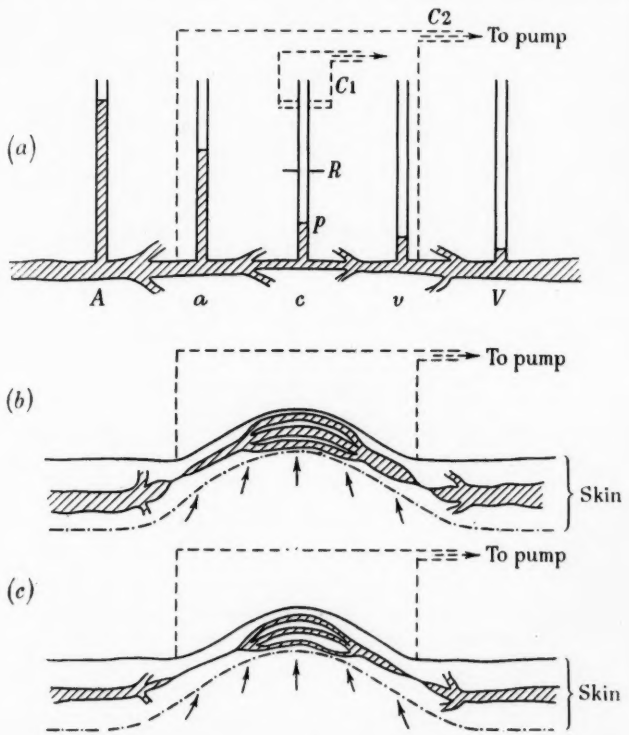


FIG. 11.—Diagrams of the hydrodynamic state of the blood vessels in the skin during cupping (p. 86). (*a*) C1 and C2, the ideal and actual position of the cup during the estimation of capillary resistance. (*b*) Distension of the capillaries in hyperaemic skin cupped at suction pressures over 100 mm. Hg. (*c*) Incomplete distension of the capillaries in ischaemic skin cupped at suction pressures over 100 mm. Hg.

skin, where the larger blood vessels lay. The various vessels were distended in the first place inversely as the thickness of their walls, and secondly in relation to the amount of blood trapped in the cupped area of skin. The distension of the vessels and the trapping of blood during the application of the cup was readily observed under a 20× magnification with a 3 mm. glass cup placed on the trans-illuminated ear of an anaesthetized guinea-pig, by gradually applying a suction of 100 mm. Hg.

The relation of susceptibility to cupping and the distribution of skin vessels.—Susceptibility to cupping in the normal guinea-pig increases with proximity to underlying larger vessels, suggesting that nearness to an easily-sucked supply of blood is necessary for distension of the capillaries. This is clear from Fig. 12, exemplifying tests on several animals. A depilated guinea-pig was cupped at regularly spaced sites on one flank for 1 min. at 200 mm. Hg and immediately killed. The skin was incised over the spine, reflected ventrally, and the main skin vessels mapped in relation to the cupped areas. The skin of the lower belly was practically insusceptible. The most consistently susceptible area lay along a line parallel to, and 15–20 mm. from the lower thoracic and lumbar spinal midline, in close relation to the vessels that emerge from the body musculature. Susceptibility was less, though marked over the sacrum, but even along

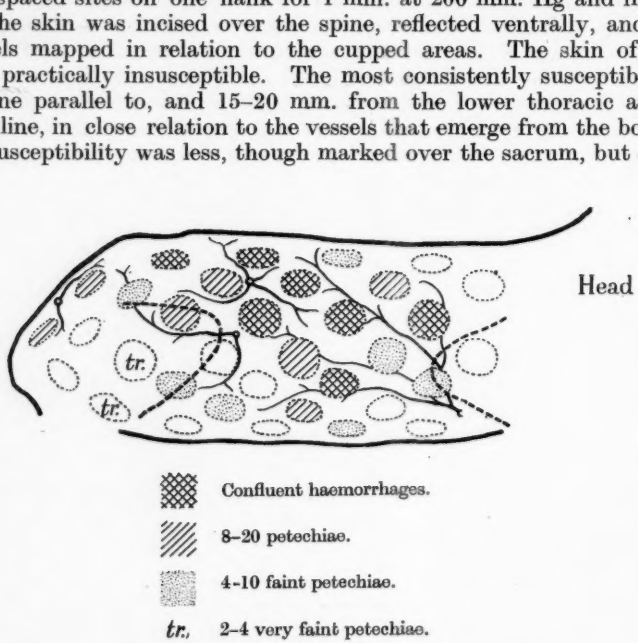


FIG. 12.—Variation of the susceptibility to capillary rupture by cupping, in relation to the branches of the main blood vessels underlying the skin of a 300 g. guinea-pig's right flank. A suction of 200 mm. Hg was applied for 1 minute to each marked area.

this line it was not uniform. Over twenty guinea-pigs were cupped in various ways and all had susceptible areas similar to those in Fig. 12. But even in such areas it was impossible to depend on any constant uniformity of response; confluent petechiae might appear in one site and none in the site immediately adjacent to it. But in nearly all the instances examined by dissection these close topographical differences were related to the position of the larger vessels on the undersurface of the skin.

For cupping tests even in the normal animal, areas of comparable susceptibility must be tested; and many sites in each animal, must be tested to allow for topographical variation. In the same animal the susceptibility was usually symmetrical about the spinal midline; and for the following tests, a line of cupped sites was first established as a control along one side of the spine of the depilated animal, and the exactly corresponding sites on the other side marked in readiness for the experiment. During marking the animal was made to sit

quietly with the spine in a straight antero-posterior line, and all distortion of the mobile skin by handling was carefully avoided.

The effect of blood supply on susceptibility to cupping.—Tested by the method outlined in the preceding paragraph, animals in a state of shock, with blood pressures of 10 mm. or less in the arteries of the ear, required cupping for 1–2 min. at 600 mm. Hg to produce petechiae similar to those made in the normal animal at 200 mm. Hg. Freshly killed animals are almost insusceptible to cupping at 600 mm. Hg. Guinea-pigs under urethane anaesthesia are susceptible, but if gradually bled dry from the carotid, become less susceptible. A simple and convincing proof of the relation consists of anaesthetizing guinea-pigs with chloroform or A.C.E. (alcohol, 1 vol., chloroform 2 vol., and ether 3 vol.); and over five-minute periods alternating a cyanotic, almost moribund state of deep anaesthesia with a light second stage. The skin susceptibility changed from almost insusceptible to normal susceptibility, and at the same time the arterial pressures in the ear alternated between 3 to 10 mm., and 20–40 mm. Hg (Table IV).

TABLE IV.—*Petechiae produced by cupping the skin of guinea-pigs for 1 minute at 200 mm. Hg., during alternating deep and light anaesthesia, compared with control sites cupped before anaesthesia.*

	Cupping.			
	Initial.		During anaesthesia.	
	Arterial pressure in ear (mm. Hg).	Petechiae* in control sites.	Petechiae in contralateral test sites.	Arterial pressure in ear. (mm. Hg).
Guinea-pig 1	28	7++	2±	10
		15++	10+	22
		C	3±	10→3**
		C	C	24
		6+	*Nil*	10→4**
		C	C	20
		8+	*Nil*	10→3**
		15+++	,,	12
		C	C	25
Guinea-pig 2	40	18+	*Nil*	4
		7+	1+	12
		10++	2±	10
		8++	*Nil*	8→4**
		C	8+	6
		1+	*Nil*	7
		C++	,,	10

* The figures indicate the number of petechiae, and ±, +, ++, +++, increasing grades in size of petechiae and intensity of colour. C = Confluent petechiae.

** Arterial pressures at beginning and end of cupping period.

By suitable adjustment of the anaesthesia, and using half-minute cupping periods, the change could be induced, and the animal allowed to revert to the normal state, within one minute. Serial sections of the resulting lesions also demonstrated the importance of well and readily filled vessels for rupture in the cup; because, although no petechiae were visible on the surface of sites cupped during the moribund state, there were a few on the underside of the skin, and these proved to result almost entirely from arteriolar, not capillary rupture. The rapid

induction and the ready reversion of the anaesthetic effect excludes the probability of a direct modification of the resistance of the capillary walls ; and the theoretical, and the other experimental considerations give no indication that anything other than variation in blood content is needed to explain the apparent change in peripheral capillary resistance during shock. We have, nevertheless, no direct proof that resistance does not rise during endotoxin shock, but we submit that there is no unequivocal evidence that it does.

We are, moreover, unable to confirm the observations of Delaunay, Pages and Cotereau (1946), that subshocking doses of endotoxin significantly increase the minimal rupturing suction. In our hands the susceptibility of guinea-pig skin to a standard suction was far too variable to allow any tests other than a semi-quantitative comparison of the degree of haemorrhage in a row of treated sites made before the intoxication, with that in the corresponding contralateral sites tested $\frac{1}{2}$, 1 and 2 hours after injection of the toxin. Only when arterial pressures in the ear were substantially lowered did the susceptibility to cupping diminish significantly. In any event, the increased capillary resistance reported with subshocking doses of toxin cannot be operative in the inhibition of diapedesis, because, as both we and Delaunay and Pages (1945) have shown, inhibition does not occur unless there is demonstrable shock.

The Effect of Blood Supply on Manifestations of Capillary Permeability.

Delaunay, Pages and Cotereau (1946) also record that the exudation of circulating trypan blue in response to substances (e.g. peptone) which increase capillary permeability, is inhibited in shock. As Landis (1946) points out, the rate of escape of dye into the tissues does not depend only on capillary permeability.

The following experiments show that transient mechanical interference with the blood supply of the skin can readily produce results similar to those following changes in capillary permeability.

1. The intradermal injection of 0·5 μg. histamine in 0·1 ml. into a normal guinea-pig with 75 mg. pontamine sky blue X per kg. body weight in its circulation, will regularly, within 2 to 3 minutes, produce a deep blue coloration of the skin 7 to 10 mm. in diameter. When the site of injection is immediately covered by a glass cup 20 mm. in diameter, and a suction of 40 to 60 mm. Hg applied to occlude the vessels at the edge of the cup, the blueing is inhibited as long as the cupping is maintained.

2. When a similar animal is mildly shocked, by colitoxin or dehydration, the blueing of the histamine lesion is retarded ; at most a pale blue spot 3 mm. in diameter appears after 10 to 15 minutes. But if the cup is applied to the injection site and a suction of 10 to 12 mm. Hg applied for 2 to 3 seconds every 10 seconds so as to promote the flow of exudate without continuously occluding the veins of the skin, substantial blueing of the histamine-treated area occurs within 5 minutes ; i.e. histamine, as in the unshocked animal, had induced an increase in the permeability of the vessels, but a diminution in blood supply had prevented its manifestation by the exudation of dye.

As an additional illustration of the dependence of cupping phenomena on a ready supply of blood, it should be noted that this manifestation of blueing by intermittent cupping, like the production of petechiae by cupping, was most readily elicited in the most vascular areas of the skin (*cf.* Fig. 12).

The Effect of Shock on Cl. welchii *and* Ps. pyocyanea *Skin Infection.*

Shock by colitoxin, hypertonic glucose and MgATP enhanced skin infection by *Cl. welchii* and *Ps. pyocyanea*, both directly and retroactively; and as with *Staph. aureus* infections, enhancement took place only with shock sufficient to lower the arterial pressure in the ear to 10 mm. Hg. Retroaction was more marked with *Ps. pyocyanea* than with *Cl. welchii; pyocyanea* lesions up to four hours old at the time of injecting the shocking agent were enhanced.

TABLE V.—*The Diameter of 24-hour-old Skin Lesions in Guinea-pigs injected Intradermally with Graded Doses of* Cl. welchii, *2 hours after Intraperitoneal Shocking Doses of Colitoxin.*

Guinea-pig.	Dose colitoxin (mg.).	Lesion-diameters (mm.) with *Cl. welchii*.					
		10^7.		10^6.		10^5.	
		I.	N.	I.	N.	I.	N.
1	7·5	25	13*	25	13*	20	17*
2	5·0	28	16*	19	12*	(4)	—
3	0·0	13	10*	(5)	—	(5)	—

I = Inflamed area, N = necrotic area.
* Lesions also spread ventrally with necrosis and oedema extending to midline.
The figures in brackets indicate slight inflammation.

A given dose of *Ps. pyocyanea*, like *Staph. aureus*, produced severe lesions with the more severe shock. *Cl. welchii* was affected rather differently; only severe shock enhanced infection by this microbe, and a given dose either was unaffected, producing a small, mildly inflamed swelling, or greatly enhanced into a large necrotic area with gravity extension down the flank and a sac of soft oedema in the anterior belly wall. The larger the dose of shocking agent, the smaller the dose of *Cl. welchii* that was enhanced in this "all or nothing" manner (Table V).

Histology of Cl. welchii *lesions.*—The histology of the *Cl. welchii* lesions was explored in the way described (p. 77) for the *Staph. aureus* lesions. The dose of *Cl. welchii* used was rather large, and in unshocked controls produced a severe lesion with necrotic centre in 24 hours. The tissue reaction during the first four hours differed from that with *Staph. aureus*. There was a progressive increase in the number of bacilli, and in collagenous degeneration and necrosis at the centre of the lesion. The lesion spread both laterally and vertically, until at four hours there was degeneration and necrosis of the superficial epithelium, and in the muscular layer immediately over the subcutaneous spaces. The necrosis affected the abundant polynuclear leucocytosis that had already taken place in half an hour, but there was, up to four hours (the oldest lesion studied), an increasingly active exudation of healthy phagocytes at the periphery of the necrotic area. In shock, the infection and necrosis spread more rapidly, and bubbles of gas appeared within one hour and were a striking feature of the older lesions, where enhancement in size was accompanied by a proportional increase both in gas and the number of clostridia; but, as with staphylococci, both fibrinous exudate and phagocytes were greatly diminished in retroactively affected lesions, and suppressed in actively affected lesions. Retroaction appeared to be much more severe than in staphylococcal lesions, presumably

because the already migrated leucocytes were more rapidly destroyed by *Cl. welchii* in the large dose used, than by the relatively small dose of *Staph. aureus*. As in the "shocked" staphylococcal lesions, the capillaries were dilated and the arterioles contracted at the periphery of the necrotic areas.

The Effect of Shock on Bact. coli *and* Pr. vulgaris *Skin Infection.*

We tested strains of two other bacteria, *Bact. coli* and *Pr. vulgaris*, which in our previous experiments had been enhanced by adrenaline. The result was surprising because the general picture was that of inhibition, not enhancement, of the infection by shock. Graded doses of the two strains were injected during

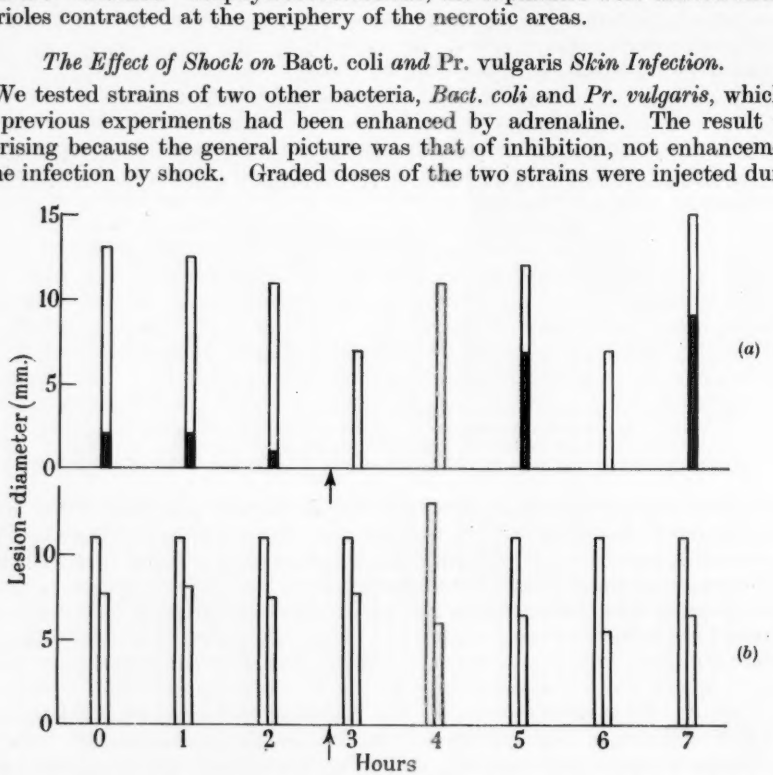


FIG. 13.—The effect of intoxication by colitoxin on lesions due to *Bact. coli* injected before and after the colitoxin. The height of the white and black columns respectively indicate the diameters of inflamed and necrotic areas. The arrows indicate the injection of the toxin. (*a*) Erratic enhancement of lesions made late in the shock. (*b*) Diminution of lesions. At each hour the left-hand columns represent lesions in one control animal, the right-hand columns the mean of lesions in two shocked animals.

the height of colitoxin shock, 2½ hours after the injection of toxin; and there was little doubt that the 24-hour lesions in the intoxicated animals were substantially smaller than those in unshocked animals (Table VI). At 24 hours the intoxicated animals had fully recovered from the colitoxin, and the infecting microbe could not be grown from the blood or the viscera. We were not therefore dealing with an absence of localized inflammatory reaction in persistently shocked or otherwise moribund animals. Nor was the result with *Bact. coli* likely to have been due to a specific effect of the colitoxin; because the colitoxin was serologically distinct from the O antigen of the infecting strain; and the antigenically unrelated *Pr. vulgaris* was similarly affected.

TABLE VI.—*The Diameter of 24-hour-old Lesions due to Graded Doses of* Pr. vulgaris *and* Bact. coli *in Guinea-pigs Shocked with Colitoxin 2 hours previously.*

Guinea-pig	Infecting microbe.	Dose of colitoxin (mg.).	Lesion-diameters* (mm.) with suspensions diluted.							
			10^{0}.		10^{-1}.		10^{-2}.		10^{-3}.	
			I.	N.	I.	N.	I.	N.	I.	N.
1	*Pr. vulgaris*	5·0	10	—	7	—	(4·5)	—	(2)	—
2		*Nil*	18	2	10·5	—	8·0	—	(5·5)	—
3	*Bact. coli*	7·5	9	3	—	—	—	—	—	—
4		5·0	10·5	5	(4)	—	—	—	—	—
5		*Nil*	19	4	12·5	—	8·5	—	(4)	—

I = Inflamed area ; N = necrotic area.

* The diameters are means of two lesions, on the right and left flanks of each animal. The figures in brackets indicate slight inflammation.

Not all *Bact. coli* lesions, however, were diminished in shock. As the experiments summarized in Fig. 13 show, although shock diminished all the lesions in two guinea-pigs (whose lesion-diameters are averaged in Fig. 13*b*), in a third animal the lesions made early in shock were diminished and those made late were enhanced. This phenomenon was observed on other occasions, though erratically. It is possible that the conditions necessary for enhancement by shock are much more critical for *Bact. coli*, and perhaps for *Pr. vulgaris*, than for *Staph. aureus*, *Cl. welchii* and *Ps. pyocyanea*. The phenomenon clearly needs investigation ; in the meantime it provides a useful check on too-ready generalizations about the nature of the enhancement in shock.

DISCUSSION.

We have demonstrated an association between enhancement of skin-infection and shock sufficient to lower the pressure in smaller arteries of the skin below a certain value ; the degree of enhancement is directly related to the suppression of leucocytosis in the infected tissues. Shocking agents differing as widely as colitoxin, *Cl. welchii* α-toxin, magnesium adenosine triphosphate, and intraperitoneal hypertonic glucose enhance according to the length of time they lower peripheral blood pressure. None of them appears to have a specific effect on the infection by *Staph. aureus* or *Cl. welchii*. Our results accord with those of Howie and Cruickshank (1947), who enhanced the lethal effect of anthrax spores in mice by the simultaneous injection, at a different site, of shocking doses of MgATP and hypertonic glucose. The relatively little enhancement observed may have been due to the spores' being injected intraperitoneally, for it is possible that in shock the vessels of the peritoneum retain their normal activity longer and to a greater degree than the vessels of the skin do.

In stressing diminished blood supply and low blood pressure as the immediate cause of a local failure of the defences against infection, we are aware that the analysis omits much that may be relevant, including humoral factors and physicochemical changes in the skin tissues. It seemed more profitable to begin with the immediate consequences of vascular change. There remains much to be settled. For example, is there in shock a complete stop in the supply to the capillaries of blood-borne leucocytes ? If the stoppage of circulation, as appears at present, is only partial, why are phagocytes not seen in the tissue sections ? For reasons given above, we are not inclined to admit the hypothesis of an increased resistance

of the capillary walls as an explanation. Moreover, our own observations provide an alternative. The absence of tissue leucocytes may be due, not to a complete suppression of diapedesis, but to a diminution in the number of leucocytes passing through the endothelium ; and if tissue leucocytosis at any moment is dynamic, in the sense that leucocytes are rapidly destroyed or removed, and as rapidly replaced from the blood, even a partial failure in replacement might soon lead to a virtual absence of leucocytes.

This explanation leaves inviolate the view that phagocytes go through the capillary endothelium under their own power. The view is generally held, but we know of no observation proving it. We do not know whether the intracellular forces behind the amoeboid movements of the polynuclear cells in relatively free conditions are sufficient to move a phagocyte through narrow holes in the capillary endothelium. It may well be that there is a critical intracapillary blood pressure required to help the leucocytes through the endothelium, either by a direct push, or by stretching the capillary wall so as to produce holes large enough for the passage of the leucocytes under their own power ; and that in shock the pressure remains for some time below this critical level. We are investigating these matters.

Our work on enhancement has so far been confined to pathogens that are neither natural infectors of the guinea-pig, nor, with the exception of *Cl. welchii*, capable in the doses used of spreading from the injection site and producing a widespread infection or toxaemia. It would therefore be unwise to speculate about the effect of shock on infection in general, especially in view of our observation that *Bact. coli* and *Pr. vulgaris* infections were sometimes *inhibited* by shock.

Nevertheless, we have established one feature of shock as an enhancer of *Staph. aureus* and *Cl. welchii* infection that deserves comment—namely, its limited retroactivity. Both *Staph. aureus* and *Cl. welchii* are common inhabitants of the immediate environment of men, and both, though especially *Staph. aureus* (see Williams and Miles, 1950) are likely to be driven into wounds at the time of their infliction. The development in the wounded man of severe traumatic shock is unlikely to inhibit the suppression of these two microbes by the naturally resistant tissues, unless it takes place within the first 2 to 3 hours after wounding ; because, if the argument from guinea-pig to man is valid, neither the coccal nor the clostridial lesion will be much modified by shock after this interval. The rarity of acute infection in traumatic shock, provided that no masses of ischaemic or dead tissue are left in the wound, probably reflects the rapidity with which the decisive tissue defences are normally mobilized.

In the guinea-pig these three-hour lesions are also insusceptible to local adrenaline (Evans, Miles and Niven, 1947). It appears that the decisive reactions both of the bacterium and the host tissues are displayed in the first three hours of infection, and determine within narrow limits the subsequent course and outcome of the disease. If this is so, the exploration of the defences against infection, as distinct from defences against the consequences of infection when it is once established, should be directed particularly to this short initial period.

SUMMARY.

Skin infections by *Staph. aureus*, *Ps. pyocyanea* and *Cl. welchii* were enhanced when guinea-pigs were shocked within 2 to 3 hours of the intradermal injection

of the bacteria. Older lesions were not affected, even by severe shock, suggesting that the outcome of these infections in the guinea-pig, is fully determined in 2 to 3 hours. *Bact. coli* infections were sometimes enhanced, sometimes diminished by shock ; *Pr. vulgaris* infections were diminished.

Enhancement was independent of the shocking agents used—bacterial endotoxins and exotoxins, magnesium adenosine triphosphate and intraperitoneal hypertonic glucose—and was associated with the absence of observable leucocytes in the infected tissue. It was produced only by shock sufficient to bring the peripheral blood pressure below the critical level represented by a pressure of less than 10 mm. of mercury in the small arteries of the ear ; and to maintain it at the low level for an hour or more.

The absence of tissue leucocytes is probably due to a diminution in the flow and the pressure of blood in the capillaries of the infected skin, resulting in a gross diminution of the supply of blood-borne leucocytes to the lesion. There was no evidence in the shocked guinea-pig of an increase in the resistance of the capillary walls to diapedesis of leucocytes.

The cupping method of measuring capillary resistance is inapplicable to shocked animals.

We are indebted to Professor H. N. Green for a supply of adenosine triphosphate, and to Dr. W. T. J. Morgan for some purified O antigen of *Sh. shigae*.

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THE BIOSYNTHESIS OF PORPHYRINS AND HAEMS BY *CORYNEBACTERIUM DIPHTHERIAE*.

J. H. HALE, W. A. RAWLINSON, C. H. GRAY, L. B. HOLT, C. RIMINGTON AND WILSON SMITH.

From the Departments of Bacteriology and Chemical Pathology, University College Hospital Medical School, the Department of Chemical Pathology, King's College Hospital Medical School, and the Wright-Fleming Institute of Microbiology, London.

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IT has been known for some time that when *C. diphtheriae* is cultured under conditions suitable for toxin production, porphyrin also appears in the culture fluid, and indeed a quantitative relationship between toxin and porphyrin was observed by Coulter and Stone (1931). The nature of the porphyrin has been studied by several workers (Campbell-Smith, 1930; Coulter and Stone, 1931; Dhéré, Glücksmann and Rapetti, 1933). Gray and Holt (1947, 1948) demonstrated, by chromatographic adsorption methods, that the major pigment is coproporphyrin III, but that there are also present small quantities of uroporphyrin I and of an unidentified porphyrin. Subsequent partition chromatographic analysis (Nicholas and Rimington, 1949) supported this conclusion, and indicated that two unidentified porphyrins are present which almost certainly contain 5 and 6 carboxyl groups respectively.

The quantitative relationship between iron content of the medium on the one hand and toxin and total porphyrin production on the other has been studied recently by Pappenheimer (1947*a*, *b*), and Pappenheimer and Hendee (1947), who state that for every four atoms of iron added over and above the optimal level for toxin production, one molecule of toxin and four molecules of porphyrin fail to appear in the culture medium. The conclusion was drawn that iron-porphyrin complex and toxin are related as prosthetic group and protein moiety in a haemoprotein fulfilling some enzymic function in the cell of *C. diphtheriae*. Although 95 per cent of the excreted porphyrin belongs to the series derived from aetioporphyrin III, there are present in the culture fluid other porphyrins, one of which is known to be related to aetioporphyrin I. This fact is not easily reconciled with Pappenheimer's theory, which postulates the building up of haemoprotein (Type III) when iron is freely available, and the excretion of the porphyrin prosthetic group and protein moiety when the iron content of the medium is restricted. Implicit in the hypothesis is the idea that the porphyrin which fails to appear in the culture medium is retained in the cell in the form of an iron complex of a Type III porphyrin, together with toxin protein. Thus it would appear possible that at higher iron levels an accumulation of coprohaem might occur within the cells. Rawlinson and Hale (1948, 1949) have investigated the nature of the haems present in *C. diphtheriae* cells and find no evidence for the presence of coprohaem; the major haemoprotein yields protohaem No. 9 (isomeric series III).

As far as pigment metabolism is concerned, the situation would thus appear to be that in the presence of abundant iron, the growing cells synthesize haemoproteins mainly derived from protoporphyrin No. 9 and pass little porphyrin into the culture fluid. When iron supply is restricted, however, less intracellular protohaem is produced and porphyrins appear in the culture fluid. Coproporphyrin III, which makes up by far the larger part of this pigment, could conceivably be either a precursor of protoporphyrin or be derived from it by metabolic transformation, but the isomerically different uroporphyrin I of the culture fluid could not possibly bear such relationships to the protoporphyrin. Alternatively it is possible that each pigment is synthesized independently, and that the appearance, according to the available supply of iron, of intracellular protohaem or of extracellular coproporphyrin and uroporphyrin, etc., is merely the expression of some redirection of the biosynthetic mechanism at a comparatively early stage in tetrapyrrol synthesis.

We considered that this system offered an opportunity to study, by the aid of isotopes, some of the questions raised. The results of our investigations are set forth below.

METHODS.

Organism.

Corynebacterium diphtheriae, Park Williams No. 8 Toronto strain, has been used throughout.

Culture media.

In Experiments 1 to 4 a synthetic medium, found by trial to be optimal for growth and porphyrin production, was used; the composition of this medium is recorded in Table I. In Experiments 5 and 6 a semi-synthetic medium was employed as described by Holt (1948), modified only by using 0·2 per cent (w/v) instead of 0·15 per cent (w/v) of glycine. The requisite quantities of iron were added as $FeSO_4.7H_2O$. The medium was distributed in quantities of 100 ml. in Roux bottles, sterilized, and after inoculation cultivated at 32° for 7 days. In Experiments 1 to 4 known amounts of glycine were added, the glycine containing an excess of ^{15}N in Experiments 2 and 4. In Experiments 5 and 6 it was necessary to determine the glycine content (method of Alexander, Landwehr and Seligman, 1945) of the casein digest prior to the addition of the isotopic amino-acid in order to calculate the final isotope dilution.

TABLE I.—*Composition of Synthetic Medium.*

		g.			*g.*
DL	Tryptophane	0·1	DL	Aspartic acid	0·5
	Glycine	1·0	L	Proline	0·6
DL	Valine	0·5	DL	Phenylalanine	0·4
DL	Alanine	0·5	L	Cystine	0·1
DL	Leucine	1·0		NaCl	2·0
L-	Glutamic acid	2·5		KH_2PO_4	0·1
DL	Methionine	0·4		$MgSO_4.7H_2O$	0·3
DL	Tyrosine	0·4			*mg.*
L	Arginine	0·3		Pimelic acid	5·0
L	Histidine	0·2		β Alanine	5·0
DL	Lysine	0·2		Nicotinic acid	10·0

Materials dissolved in 500 ml. distilled water with the exception of cystine, which was dissolved in minimum of *ca.* 5N HCl and then added. pH adjusted to 7·6, volume made up to 900 ml., and after the addition of 3 ml. of 3·3 per cent (w/v) $CaCl_2$ boiled 15 min. and filtered. $MgSO_4$ and tryptophane added, made up to 1000 ml. sterilized by autoclaving. 3·5 ml. 45 per cent (w/v) maltose solution and 4 ml. of 0·001 per cent (w/v) $FeSO_4.7H_2O$ added per 90 ml. medium.

Harvesting.

The cultures were centrifuged and the cells washed three times with 10 vol. of 0·9 per cent (w/v) NaCl. They were again resuspended in saline and portions removed for determination of total N and ^{15}N excess. The remainder was used for total haem extraction. The supernatant fluid, after determination of Lf and total coproporphyrin content, was treated with formaldehyde, to convert the toxin to toxoid.

Isolation of the porphyrins from the toxoid solution.

This was achieved by methods described by Gray and Holt (1948). In the case of experiments with high iron levels it was not possible to effect isoelectric precipitation. The coproporphyrin was then extracted with ether, and the material that separated at the interface was worked up for uroporphyrin. The porphyrins were subjected to chromatographic purification after esterification.

Isolation of protoporphyrin from intracellular haems.

The haems extracted from the washed cell residue by the method described by Rawlinson and Hale (1949) were adsorbed from the ethereal solution upon an alumina column, eluted by glacial acetic acid and converted to porphyrins by the method of Warburg and Negelein (1932). The porphyrins were taken into ether and the protoporphyrin removed by shaking with 5 per cent HCl. The porphyrin of the cytochrome *a* complex remained in the ether, but was present in too small a quantity to make isolation practicable. The protoporphyrin was purified by repeated transfers between acid and ether.

TABLE II.—*C. diphtheriae. Large*

Experiment.	Total Glycine N in culture fluid (mg.).	^{15}N in Glycine (atom % excess).	Total Bact. N (mg.).	^{15}N in Bact. Protein (atom % excess).	Lf.
(1) Low Fe* normal glycine	858·4	*Nil*	475	*Nil*	14
(2) Low Fe* ^{15}N glycine	678·5	5·584	487·5	0·903	20
(3) High Fe* normal glycine	522·5	..	560	0·008	4–5
(4) High Fe* ^{15}N glycine	678·5	5·584	632·5	0·863	12
		Large Experiment 2 (20 *litre/batch*).			
	(g.)		(g.)		
(5) Low Fe* ^{15}N glycine	7·40	4·92	9·25	0·790	55
(6) High Fe* ^{15}N	7·14	4·92	8·57	0·729	5

* Low Fe, opt. iron for toxin production 0·147 μg./ml. High Fe = 5 × opt. iron.

Isotope analysis.

All materials were digested for 24 hours with H_2SO_4 containing 2 per cent (w/v) SeO_2, copper and potassium sulphates being added as additional catalysts. Where a specimen contained insufficient nitrogen (*ca.* 0·25 ml.) for the mass spectrograph dilution was effected with some of the normal substance, the relative proportions being determined spectrophotometrically. All ^{15}N analyses were kindly carried out by Dr. R. Bentley at the National Institute for Medical Research.

RESULTS AND DISCUSSION.

The results of the experiments are presented in Table II. The isotope enrichment of both protein fractions and haems or porphyrins is seen to be little affected by the nature of the basal medium, i.e. either casein digest or mixture of synthetic amino acids respectively. In each case the labelling of the intracellular haem is higher than that of the bacterial protein, whilst that of the coproporphyrin III is distinctly lower. In the Experiments 5 and 6, where isolation of uroporphyrin I was also possible, the labelling of this porphyrin was approximately the same as that of the coproporphyrin.

The ways in which the coproporphyrin of the culture fluid might be expected to be formed are :

(1) By independent synthesis from a nitrogenous precursor or precursors different from that used for synthesis of the protoporphyrin of intracellular haem.

(2) By an independent synthesis from the precursor of the intracellular haem.

(3) By a route in which coproporphyrin III is a precursor of protoporphyrin or vice versa (Fig. 1).

(4) By metabolic transformation from the protoporphyrin of the intracellular haem.

The last of these is unlikely, because the coproporphyrin excreted into the culture medium cannot have been derived directly by some metabolic transformation from the protoporphyrin of the intracellular haems or vice versa, otherwise a comparable degree of labelling would have been found.

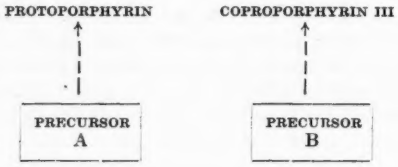
Experiment 1. (*Synthetic Medium.*)

^{15}N in Toxoid Protein (atom % excess).	^{15}N in Non-toxoid Protein (atom % (excess).	^{15}N in Intracellular Haem (atom % excess).	Copro-porphyrin (mg.).	^{15}N in Copro-porphyrin (atom %) excess).	Uropor-phyrin (mg.).	^{15}N in Uropor-phyrin (atom % excess).
Nil	*Nil*	..	7·0	0·023	—	—
0·88	0·88	..	9·9	0·489	..	..
..	..	..	2·2	..	..	..
..	..	1·247	8·2	0·511	..	..

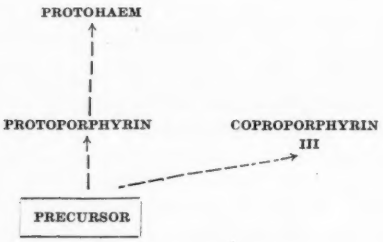
(*Casein digest Medium.*)

..	..	38·0	0·510	approx. 0·8	0·463
..	1·261	3·2	0·284	approx. 0·4	0·342

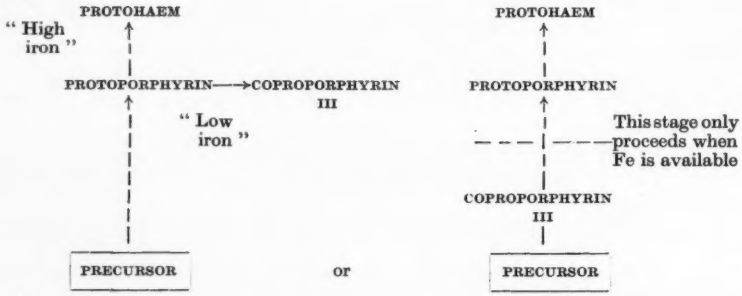
Scheme 1.



Scheme 2.



Scheme 3.



Scheme 4.

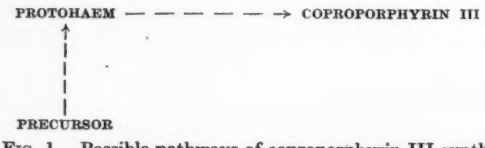


FIG. 1.—Possible pathways of coproporphyrin III synthesis.

At present there exists no conclusive experimental evidence concerning the specific nitrogenous precursors of coproporphyrin or uroporphyrin, although coproporphyrin I and uroporphyrin I excreted by a case of congenital porphyria have been shown by Grinstein, Aldrich, Hawkinson and Watson (1949) and Gray and Neuberger (1949) to be labelled to a high degree after the feeding of isotopic glycine to the subject. In the case of *C. diphtheriae* a different coproporphyrin isomer is concerned. In our experiment uroporphyrin belonging to the series I could not have been derived directly from the coproporphyrin III or vice versa, but the fact that the isotope concentration in these two substances was practically equal suggests that they may have been derived from the same nitrogenous precursor.

With regard to the possibilities (2) and (3), synthesis of coproporphyrin may occur at a later time during cultivation than that of protohaem. Protohaem

would most probably be formed while the isotope content was maximal, coproporphyrin possibly only after protohaem synthesis had decreased through reduction of the iron content of the medium. The isotope content of the glycine in the medium might by then have been lowered by randomization. In this respect separate experiments have shown that the growth of *C. diphtheriae* is independent of glycine, the organism being capable of synthesizing this amino acid. On the other hand, porphyrin production per mg. of bacterial nitrogen is slightly higher in cultures to which an external source of glycine has been added. Consistent with the hypothesis that coproporphyrin III is formed at a later stage than protohaem is the fact that the isotope content of the coproporphyrin in Experiment 5 with "low iron" is much higher than that in Experiment 6 with "high iron." In the latter experiment one may assume that protohaem production continued for a longer period than when less iron was available, so that the coproporphyrin production in this case was delayed until randomization had lowered the isotope concentration of the glycine. Unfortunately isotope analyses of the glycine in the medium were not carried out at intervals during the growth of the organism.

Our observations are therefore equally compatible with the possibilities (1), (2) and (3) listed above. The first hypothesis although a possibility is, we feel, less likely in view of the abundant reported evidence for the participation of glycine in the biosynthesis of the porphyrin ring.

SUMMARY.

The incorporation of ^{15}N glycine into the proteins, porphyrins and haems elaborated by *C. diphtheriae* has been studied.

Glycine is incorporated into all these compounds, but the degree of labelling differs. This is discussed and hypotheses are advanced to account for these findings.

The isotopes supplied were kindly made available by the Tracer-elements Sub-Committee of the Medical Research Council, and we are greatly indebted to Dr. R. Bentley for performing the isotopic analyses.

We wish to thank Mr. G. D. Clarke of the Wright-Fleming Institute for bacterial nitrogen and toxoid estimations, and Mrs. E. Work of the Chemical Pathology Department, University College Hospital Medical School, for paper partition chromatographic analyses of the synthetic culture media.

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SOME ANTIBIOTICS FORMED BY *BACTERIUM COLI*.

JOAN F. GARDNER.

From the Sir William Dunn School of Pathology, Oxford.

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THE literature of the past 50 years concerning antibiotic production among the Gram-negative intestinal bacilli has been reviewed in detail by Florey, Chain, Heatley, Jennings, Sanders, Abraham and Florey (1949). The recent work of Fredericq, Gratia and their co-workers has resulted in the recognition of more than 17 distinct types of colicine, one or more of which may be produced by any one strain of *Bact. coli*. Their classification is based on such properties as the character of the zone of inhibition on solid media, the rate of diffusion in agar, the rapidity of destruction by bacterial proteases, and especially on their behaviour towards a series of artificially-selected resistant mutants. A thesis by Fredericq (1948) contains a full account of the methods used and the results obtained. The investigations to be reported in this paper were undertaken in order to select strains known to produce different colicines, to develop methods for their preparation, concentration and partial purification, and then to compare some of their properties.

EXPERIMENTAL.

Detection of Antibiotic-Producing Strains of Bact. coli.

Source of cultures.

The majority of the strains tested were isolated originally from specimens of human faeces submitted for routine bacteriological examination. A small number were isolated from urine and one from sewage. Few data were available concerning the characteristics of the majority of these organisms.

Seven strains previously shown to be antagonistic to various Gram-negative bacilli had been maintained in the laboratory for some time. They included typical faecal coli strains, i.e. *Bact. coli* Type I, in addition to atypical *Bact. coli* Type I, and *Bact. coli* Type II. Another series of 36 strains of known biochemical and serological characteristics was made available by Dr. J. Taylor.

Method of detecting antibiotic production.

The cross-streak method on agar plates proved the most reliable. Five to ten per cent of fresh blood was included in the medium as a source of catalase in order to eliminate any peroxide which might be formed, since only the more specific inhibitors were of interest in this investigation. The strain to be tested was streaked across the diameter of the plate, which was incubated for 24 hours before the test organisms were streaked on either side at right angles. The multiple streak method of Heatley (1947), in which the primary streaks were

separated from the agar surface by a layer of cellophan which was stripped off before making the secondary streaks, was also used. However, some antibiotics would have remained undetected by this method because of their inability to pass through cellophan. Increasing the porosity by immersion in zinc chloride solution (Seymour, 1940) did not completely eliminate this possibility, and difficulties were encountered in the preparation and manipulation of the treated cellophan. The test organisms used included *Sh. sonnei*, *Sn. shigae*, *S. newport*, *S. paratyphi A* and 8 strains of *Bact. coli*. Most of the antibiotics formed by *Bact. coli* are known to be nighly specific, and it was realized that, however wide the range of test organisms used, some might remain undetected because no organism susceptible to them was included in the series.

Results of survey.

Of a total of 317 strains tested for antibiotic production by the cross-streak method, 37 were found to inhibit a varying number of the test organisms. This proportion is in agreement with the rate of incidence reported by Fredericq and Levine (1947*a*, 1947*b*), and by Halbert (1947, 1948*a*, 1948*b*). The active strains were examined by Dr. Fredericq, who classified the colicines according to his system. He reported that 27 organisms produced the colicine V of Gratia (1925), while 3 produced this colicine and one other, the latter being different in the 3 cases but not identified. Two produced colicine D alone, inhibitors distinct from the 17 of Fredericq's original classification were produced by 2 strains, and 3 produced colicines in such low concentration that identification was not possible. Some of the data relating to the range of action of the colicines in this series are shown in Table I. The only colicine V-producing strains which are included are those which show differences in their fields of antibacterial action. These mainly concern activity against *Sh. shigae*, *S. paratyphi A* and *Bact. coli* Strain No. K 19227, and may have been quantitative rather than qualitative.

Selection of strains for further investigation.

Three organisms, each producing a different colicine, were selected from the limited series available. For the investigation of colicine V, Strain No. 17380, a typical *Bact. coli* Type I, was selected because its cultures in broth were not viscous and, unlike some of the other strains producing this colicine, could be easily clarified. Of the 2 strains producing colicine D, Strain No. K 19227 was selected because it inhibited the more strongly. Strain No. A 20*a*, which produced a distinct but unclassified antibiotic, was selected as the third strain. For convenience these three organisms will be referred to as Strains 1, 2 and 3 respectively.

Production of Colicines in Liquid Media.

Method of assay.

The cylinder-plate method (Abraham, Chain, Fletcher, Florey, Gardner, Heatley and Jennings, 1941) was used, appropriate sensitive strains of *Bact. coli* being used as test organism. For colicine D, *Bact. coli* NCTC No. 86 was the most suitable, while the sensitive strain described by Heatley and Florey (1946) and known in the laboratory as " selected *Bact. coli* " was used for assaying

TABLE I.—*Range of Activity of Strains of* Bact. coli *Determined by Cross-streak Tests on Blood Agar.*

++ denotes inhibition to a distance of 7 mm. or more from the primary streak.
+ denotes inhibition to a distance of less than 7 mm.
± denotes only a trace of inhibition, or partial inhibition.

Active strain Number.	Colicine produced.*	Degree of inhibition when tested against—											
		Bact. coli.								*Shigella.*		*Salmonella.*	
		Selected lab. strain.	NCTC No. 86.	Strain "Wiedling."†	No. K19227.	No. 19398.	NCTC No. 693.	NCTC No. 1092.	NCTC No. 1094.	*sonnei.*	*shigae.*	*newport.*	*paratyphi A.*
17380 ("Strain 1")	V	++	−	++	++	++	++	−	++	++	++	−	+
A49*b*	V	++	−	++	−	++	++	++	++	++	−	−	−
A18*d*	V	++	−	++	+	++	++	++	++	++	++	−	++
A473	V	++	−	++	++	++	++	++	++	++	±	−	++
19396	V	++	−	++	++	++	++	++	++	++	++	+	++
U5–41	V and one other‡	++	±	++	±	++	++	++	+	++	−	−	++
NB15BA	V and one other‡	++	−	++	−	++	++	++	−	++	+	−	+
V103	V and one other‡	++	−	++	−	++	+	+	−	++	−	−	−
V106	D	+	++	+	−	+	+	+	+	−	−	−	−
K19227 ("Strain 2")	D	+	++	++	−	+	+	+	+	±	±	−	−
U14–41	Distinct from known colicines	+	−	−	−	−	−	−	−	+	−	−	−
A20*a* ("Strain 3")	Distinct from known colicines	++	−	+	−	−	−	−	−	++	−	−	−
19398	Not classified	−	−	−	−	−	−	−	−	−	±	++	−

* Identified by Dr. P. Fredericq on the basis of his system of classification.
† Described by Wiedling (1945).
‡ These unidentified colicines were distinct from one another.

the other colicines. To avoid unduly large variation it was necessary to standardize the density of the bacterial suspensions with which the plates were seeded. With the antibiotic produced by Strain 3 it was advantageous to keep the plates (with cylinders filled) at room temperature for some hours before incubation was commenced, as the zones of inhibition were otherwise so small. Because of the tendency for resistant colonies to develop, single susceptible colonies were isolated at intervals and used as stock cultures.

No suitable standard was available for these antibiotics, but each was tested in a series of dilutions, and attention was paid to the highest dilution at which inhibition was observed. Since the curves relating zone diameter to concentration were very flat, fourfold and sometimes sixteenfold dilutions were prepared.

Culture methods.

Cellophan sac cultures were prepared as described by Heatley and Florey (1946).

Shallow stationary cultures were grown in the flat porcelain vessels originally used for penicillin (Abraham *et al.*, 1941), the depth being varied from 0·25–1 cm. The optimum depth of medium and time of incubation for antibiotic production were found to vary with the different strains.

Growing the organism on the surface of shallow layers of medium containing 0·5 per cent agar (Halbert and Magnuson, 1948) failed to give extracts of greater antibiotic potency than that attained in liquid culture.

Aerated cultures were set up in glass tubes 18 in. by 2 in., fitted with sintered glass air dispersers. Such factors as rate of aeration, composition of the medium, strength of buffer salts and antifoam agents were varied. Lard oil containing 1 per cent undecanol proved the most effective antifoam agent.

Media.

Antibiotic production occurred readily in Lemco broth, but the probable protein or peptide nature of the colicines rendered a medium free from precipitable protein desirable. Good growth, but no antibiotic-production, occured on media containing a digest of casein. Production was satisfactory, however, on a synthetic medium containing ammonium lactate and inorganic salts, based on that described by Halbert and Magnuson (1948), but the inclusion of peptone resulted in a more rapid rate of growth and production.

Lemco broth.		*Paptone-lactate medium.*	
Lab. lemco	1·0 per cent	K_2HPO_4	3·0 g.
Eupepton	1·0 ,,	KH_2PO_4	1·5 g.
NaCl	0·5 ,,	$MgSO_4$	0·2 g.
Made up with tap water.		NaCl	2·0 g.
		Lactic acid*	10 ml.
		Evans' peptone	5·0 g.
		Tap water	1 litre.

* Neutralized with sodium hydroxide unless peptone was omitted from the medium, when ammonium hydroxide was used instead.

Culture experiments were carried out also in variations of the simpler medium. In one the peptone was omitted, another contained peptone but no lactic acid,

while a third contained peptone with lactose substituted for the lactic acid. No antibiotic was produced on media containing glucose.

Using the various media and culture methods outlined in the preceding paragraphs, the optimal conditions for the production of antibiotic by each of the three strains chosen for study were ascertained, and sufficient of each was prepared in a crude form for an investigation of its properties.

Strain 1 produced readily on cellophan sacs and in layers of liquid medium up to 1 cm. in depth. In Lemco broth and in peptone-lactate medium maximum activity was attained in 24 hours at 37° C., while in lactate medium without peptone 3 days' incubation was necessary. In peptone medium without lactic acid the activity was lower, and addition of lactose decreased it further.

Strain 2 likewise produced readily on cellophan sacs, the yield increasing to a maximum during the first 3 days as successive daily replacements of the medium were made. In shallow layers of peptone-lactate medium activity reached its highest level in 36 hours if the depth did not exceed 0·5 cm. In deep, aerated culture a similar level of activity resulted in 10 to 12 hours, after which it declined until the activity finally disappeared. This organism was the only one which produced a detectable amount of antibiotic when the cultures were aerated. A relatively slow rate of aeration (0·5 volume of air per volume of medium per minute) gave the best results.

Strain 3 produced little activity on cellophan sac cultures and none in aerated cultures. In stationary cultures 0·5 cm. deep activity resulted after 18 hours' incubation, but thereafter declined rapidly.

Concentration from the culture medium.

The preliminary treatment of the culture fluid varied slightly. Cultures of Strain 1 could be readily clarified without loss of activity by adjusting to pH 3 and allowing the precipitate to settle, whereas cultures of the other organisms were not clarified by this process and some activity was lost in the case of Strain 2. The bacteria were therefore removed as completely as possible by passing cultures of Strains 2 and 3 through a Sharples centrifuge at neutral pH.

The antibiotics were precipitated from the substantially bacteria-free culture fluids by addition of 55 g. of ammonium sulphate to every 100 ml.

The precipitate was re-dissolved in a small volume of water and dialysed to eliminate ammonium sulphate. The dialysed solution contained much suspended material which was not entirely removed by centrifuging. Addition of calcium chloride and sodium phosphate solutions in small amounts produced a precipitate which carried down some of the suspended material without decreasing the activity. Some inactive material could also be coagulated by heating, in the case of the antibiotics which were heat-stable. The solution was finally dried from the frozen state, yielding a non-hygroscopic product which could be stored in the refrigerator without loss of activity. The most active preparation obtained was from Strain 1, grown on ammonium lactate medium, 30 mg. of the dried product being obtained per litre of culture fluid.

Properties.

The majority of the tests were carried out with concentrated solutions of the dried antibiotic preparations (20–30 mg./ml.), the remainder being performed with the clarified culture fluids. Drying from the frozen state or in a current

of warm air did not cause any loss of activity. The results indicate colicine D to be considerably less stable than the others investigated, though there did not appear to be any pronounced difference between the properties of colicine V and the one produced by Strain 3.

A preliminary examination of the antibiotic produced by Strain 3 had already been made by Heatley (1948, unpublished), who found that it could be produced in stationary liquid culture under certain conditions, but not in deep aerated cultures. The active substance was stable to boiling in neutral or acid solution and was unaffected by organic solvents, though it was insoluble in those tested, with the exception of glacial acetic acid and pyridine. It was destroyed by proteolytic enzymes. It remained in solution when the culture was acidified, but was adsorbed on charcoal, from which it was not readily eluted. Tannic acid precipitated the antibiotic from solution, but it proved impossible to decompose the precipitate and recover the activity. The molecular size was assumed to be fairly large, for when the organism was grown on cellophan on agar the antibiotic did not readily pass through the membrane and it diffused only slowly in agar.

Effect of variations in temperature and hydrogen ion concentration.

Colicine V was stable in solution at room temperature at all pH values from 1 to 10, withstood heating to 100° C. for 2 hours between pH 3 and pH 8·5, and was scarcely affected by autoclaving at 120° C. for 20 minutes at pH 7. On boiling at pH 1 inactivation was complete in one hour, and in a shorter time at pH 10.

Colicine D was stable in solution at room temperature between pH 2 and pH 9. Loss of activity was detectable when alkaline solutions were held at 50° C. for 2 hours, and was very marked in half an hour at 70° C. at all pH values, 75 per cent or more of the activity being destroyed.

The antibiotic from Strain 3 was stable to boiling for 15 minutes at pH 3–5, but at pH 7 slight inactivation was noticeable, while at pH 9 only 25 per cent of the original activity remained. Little or no destruction occurred at room temperature between pH 3 and pH 9.

Action of proteolytic enzymes.

A commercial trypsin preparation was used. Solutions of colicine V were inactivated by 0·1 mg. per ml. within 15 minutes at 37° C. Commercial pepsin (2 mg. per ml.) inactivated the same solution in 30 minutes, though at lower enzyme concentrations some activity still remained.

Colicine D was also inactivated, though less readily, 0·5 mg. of trypsin per ml. being required to effect complete destruction in 15 minutes, while 10 mg. of pepsin per ml. only caused 90 per cent inactivation.

The colicine produced by Strain 3 resembled colicine V in the readiness with which it was inactivated by trypsin, though it was somewhat more resistant to pepsin.

Strictly quantitative comparisons are not possible owing to impurity of the colicine preparations. The destruction of colicine D by trypsin is contrary to the reports of Gratia and Fredericq, who consider it totally resistant. However, their tests were carried out under different conditions and the concentration of trypsin used was not stated.

Surface inactivation.

Each colicine lost about 75 per cent of its activity when nitrogen was bubbled through the solution for 3 to 4 hours.

Filtration.

When passed through asbestos filter pads or gradocol membranes the 3 preparations yielded inactive filtrates. A sintered glass filter also held back the activity on some occasions, though solutions which were initially clear could be passed through it without loss.

Dialysis.

None of the colicine preparations was dialysable through cellophan. This was surprising in view of their production within cellophan sacs, and the apparent passage of many of the colicines through cellophan in multiple streak tests. It is possible that the effect is purely quantitative, for the concentration gradient in such experiments may be many times greater than was attainable in the simple dialysis experiments. It is also possible that some increase in molecular size of the antibiotic occurs subsequent to its formation.

Organic solvents.

When the dried preparations of colicine V and of the one formed by Strain 3 were stirred with ethyl alcohol, acetone, chloroform, ether, petroleum ether, ethyl acetate, benzene, butyl alcohol or glacial acetic acid no activity was lost. The active substances were insoluble in all of these solvents except glacial acetic acid, 80 per cent ethyl alcohol, and 80 per cent acetone.

Colicine D did not differ from these with regard to solubility, but it was rather unstable in the presence of most solvents.

Precipitation.

Concentrated solutions of each antibiotic gave a precipitate when the pH was lowered to 3–4 by addition of hydrochloric acid, but the activity of colicines V and D was distributed between the precipitate and the remaining solution. With the colicine from Strain 3, however, the precipitate contained all the activity, and could be re-dissolved to give a solution much clearer than the original.

Alcohol, in concentrations up to 95 per cent, gave precipitates which were almost devoid of activity, except in the case of colicine D, where the separation was less complete. The activity could be recovered from the alcoholic solution after evaporation to dryness, but except when carried out on a very small scale the yield was too variable for the method to be used as a preparative procedure. The three antibiotics were precipitated by 90 per cent acetone, though some loss occurred.

They also behaved similarly in being quantitatively precipitated by trichloracetic and phosphotungstic acids, while metaphosphoric acid caused only partial precipitation with some loss. Picrates were formed which contained a variable proportion of the original activity. Lead acetate precipitated all the activity from the culture fluid, but no activity could be recovered from the precipitate.

Range of antibacterial activity.

This was investigated by a radial streak method, in which a solution of the antibiotic preparation was filled into a cylinder placed in the centre of a heart-agar plate. After 24 hours in the refrigerator to allow time for diffusion, radial streaks of various test organisms were made, beginning close to the cylinder. After 24 hours' incubation inhibitions were measured in terms of the distance in mm. from the edge of the cylinder to the beginning of growth of the streak. Of the limited number of bacteria tested against the three colicines, only the Gram-negative intestinal bacilli were inhibited. The range and extent of activity of the preparations are shown in Table II, and correspond closely with those shown by the living organisms from which they were produced, except in the case of the colicine from Strain 3, the potency of which was very low.

TABLE II.—*Range of Antibacterial Activity by the Radial Streak Method, Measured in Terms of the Extent (in mm. from the Edge of the Cylinder) of Complete and Partial Inhibition of the Test Organisms.*

Diffusion was allowed to take place for 24 hours at 4° C. before streaks of the test organisms were made. The figures in brackets represent the total distance of complete and partial inhibition.

Organism.	Colicine V (from Strain 1), 50 mg./ml.	Colicine D (from Strain 2), 80 mg./ml.	Colicine from Strain 3, 80 mg./ml.
Bact. coli selected strain	8 (11)	5·5	(5)
,, ,, NCTC No. 86	0	8	0
,, ,, "Wiedling"	5 (10)	4·5	0
Sh. sonnei	5 (10)	4·5	0
,, *shigae*	4 (7)	3·5	0
S. paratyphi A	(7)	0	0
,, *enteritidis* (Gaertner)	0	0	0
Bact. coli No. K 19227	(5)	0	0
,, ,, No. 19398	5·5 (10)	2·5	(6)
,, ,, NCTC No. 693	6 (9)	4	(4)
,, ,, ,, No. 1092	4 (11)	6	(7)
,, ,, ,, No. 1094	4 (6)	0	0
Staph. aureus	0	0	0
B. anthracis	0	0	0
C. xerosis	0	0	0
Myco. phlei	0	0	0

Serial dilution tests in liquid medium.

Colicine D could not be satisfactorily tested in this way owing to the difficulty of obtaining a sterile solution without destroying the activity, but the titre of the other colicines against the selected *Bact. coli* was estimated. A solution containing 10 mg. per ml. was sterilized by immersion in boiling water for 5 minutes. Serial dilutions were made and inoculated with 1 drop of an overnight broth culture of the organism diluted 1 in 1000 or 1 in 1,000,000. A similar test was carried out in the presence of 10 per cent serum. The results were read at

3-hour intervals, beginning 7 hours after inoculation, because it was anticipated that resistant organisms might grow up and mask the result.

The results, shown in Table III, show that the titre was influenced to some extent by the size of the inoculum, and that the presence of serum raised it. The latter was surprising, but a similar result was reported by Heatley and Florey (1946) in the case of the antibiotic produced by *Bact. coli* strain CF1.

TABLE III.—*Activity Measured by Serial Dilution Test in the Presence and Absence of Serum and with Different Inoculum Sizes.*

Time of incubation (hours).	Inoculum : broth culture diluted 1 part in 1000.				Inoculum : broth culture diluted 1 part in 1,000,000.	
	No serum.		10 per cent serum.		No serum.	
	Colicine V from Strain 1.	Colicine from Strain 3.	Colicine V from Strain 1.	Colicine from Strain 3.	Colicine V from Strain 1.	Colicine from Strain 3.
7	562,000	4000	1,000,000	8000	No growth	No growth
10	256,000	2000	562,000	8000	1,000,000	8000
13	256,000	2000	562,000	8000	562,000	4000
16	64,000	2000	256,000	8000	256,000	4000
48	16,000	1000	128,000	4000	64,000	1000

SUMMARY.

Of a series of more than 300 strains of *Bact. coli* which were investigated for antibiotic production by cross-streak tests, 37 were found to inhibit a varying number of the test organisms used. The latter were chiefly strains of *Bact. coli*, but a few strains of *Shigella* and *Salmonella* were also included. Three active organisms which produced colicines distinct from one another on the basis of Fredericq's classification (Fredericq, 1948) were selected for more detailed investigation.

The active agents were all produced most satisfactorily in shallow stationary liquid cultures of the organisms, the most suitable medium being a solution of ammonium lactate and inorganic salts, with or without the addition of peptone, which resulted in more rapid growth and antibiotic production. They were precipitated from the bacteria-free culture fluids by ammonium sulphate and the dialysed solutions dried from the frozen state.

The 3 colicines did not differ markedly from each other with regard to chemical properties. All appeared to be protein or peptide in nature, and susceptible to the action of pepsin, trypsin and papain. Colicine D was distinguished from the other two by the ease with which it was destroyed on heating in aqueous solution and its lability on contact with organic solvents. All these substances remained in solution in the presence of 95 per cent ethyl alcohol, but were precipitated by trichloracetic and phosphotungstic acids.

The range of antibacterial activity of the colicine preparations as determined by radial streak tests corresponded with that determined initially by the cross-streak method. It differed markedly in the three cases, all the organisms inhibited being Gram-negative. The activity as measured by serial dilution tests in broth was increased to some extent by a decrease in the inoculum size, and it was also increased by the presence of serum.

The properties of these antibiotics render therapeutic application improbable.

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THE ISOLATION OF THE BACTERIOPHAGES OF *PS. PYOCYANEA.**

P. T. J. C. P. WARNER.

From the Bland-Sutton Institute of Pathology, Middlesex Hospital, London, W.1.

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BACTERIOPHAGES or bacterial viruses of *Ps. pyocyanea* have been comparatively little studied, but Okuda (1923), Lisch (1924), Hadley (1924), Ashesova (1926), Gaté and Gardère (1927), Jadin (1932), Rabinowitz (1934), Linz (1938), Fastier (1945) and Dickinson (1947) have each described one or a few filtrates which produced the well-known plaques, typical of bacteriophage action, on suitable sensitive strains of *Ps. pyocyanea*. These filtrates were obtained from sewage or from broth cultures of strains of *Ps. pyocyanea*, such strains being known as lysogenic.

In this paper the term bacteriophage is only applied to agents such as have been described by the above workers, which can be obtained in a fluid free from organisms and can, in suitable concentrations, produce plaques on a confluent surface growth of *Ps. pyocyanea*. It will not cover the apparently autolytic phenomenon usually associated with an iridescent appearance which, for the sake of clarity, will be referred to as "iridescent clearings."

This paper describes a simple method of isolation of the bacteriophages of *Ps. pyocyanea* from lysogenic strains, the appearances produced by these bacteriophages on sensitive strains of the organism, and the conditions under which they are best propagated. This work was a necessary preliminary to the study of the nature of the iridescent clearings, referred to above, and which will be dealt with in a subsequent paper. It also opens the way for the identification of strains of *Ps. pyocyanea* by bacteriophages in the same manner as the well-known bacteriophage typing of typhoid bacilli and staphylococci. This may become important because *Ps. pyocyanea* is commonly associated with wound infection, and is refractory to most chemotherapeutic agents except, in some cases, streptomycin.

In addition it opens a convenient field for the study of the bacteriophage-bacterium relationship, because *Ps. pyocyanea* is not nutritionally exacting and will grow in very simple synthetic media (Robinson, 1932).

MATERIALS AND METHODS.

The 68 strains of *Ps. pyocyanea* which were used in this investigation were obtained from infected urines, infected surface wounds, "sticky" eyes of newborn infants, and faeces from infantile diarrhoea, from patients in the Middlesex Hospital; Stoke Mandeville Hospital, Buckinghamshire; and Queen Charlotte's

* This paper formed part of a thesis approved by London University for the degree of M.D.

Maternity Hospital; two were obtained from dust in Nottingham (kindly provided by Dr. Dickinson of Messrs. Boots), and two from laboratory stock cultures.

All strains were Gram-negative bacilli which produced a diffusible green pigment on solid media and had the cultural characteristics of *Ps. pyocyanea*. In addition the majority of the strains fermented glucose.

The strains were kept in broth agar stab cultures, as basic stock cultures, and on broth agar slopes as "working" stock cultures, the latter being used as required and renewed as growth was removed in the course of the work.

All 68 of the strains were tested for lysogenicity (the property of carrying bacteriophage), and 65 of them were tested for sensitivity to the bacteriophages isolated. Thus most of the strains of organisms were subjected to two distinct series of tests, both being performed concurrently. For the purpose of clear description it is supposed that there are two groups of strains, those that were tested for lysogenicity and those that were tested for sensitivity to bacteriophages.

The 65 strains of *Ps. pyocyanea*, tested for sensitivity to bacteriophage action, were dealt with in batches; when a bacteriophage preparation was isolated active against a particular strain in the first batch, this strain was put on one side and not tested again. The active bacteriophages isolated at this time, when fully potent, were then tested against another series of strains, any of which, if sensitive to bacteriophage action, were also put on one side; those that were not sensitive together with those strains from the first batch, against which no bacteriophage had been found active, constituted the second batch, which therefore consisted only of strains of *Ps. pyocyanea* insensitive to the bacteriophages so far isolated. This ensured that, when a further bacteriophage preparation was found active against a strain in the new batch, the range of activity of such a preparation was different from previous ones, and therefore had a different bacteriophage content. The procedure was repeated until sufficient strains were found sensitive to the bacteriophage preparations collected at that time.

Not infrequently two or more bacteriophage preparations showed activity against the same organism. Only the most suitable one of these was retained, the others being stored for later study.

The strains which were tested for lysogenicity were also dealt with in batches which did not coincide with those used in testing for sensitivity. Each batch used was tested for the presence of bacteriophages active against one of the several batches of organisms being tested for sensitivity.

This system was adopted because the organisms were collected over a period of time, and were examined as they were received.

Method of preparation of bacteriophage suspensions.

The first 38 strains were tested for lysogenicity by conventional methods which differed little from those described by workers from the time of Lisbonne and Carrère (1922) to the present day. Broth cultures of the strains being tested for lysogenicity were passed through Seitz filters, and the filtrates thus obtained tested against surface cultures of strains being tested for sensitivity to bacteriophages.

The method used with the 30 strains obtained subsequently is described in

detail, because it differs in several particulars from classical methods and has met with most success when used exactly as described:

Screw-capped 1-ounce bottles containing peptone water, in 5 to 10 c.c. quantities, were inoculated with a number (23, 4 and 3 on separate occasions) of strains of *Ps. pyocyanea* and incubated for about 6 hours at 37° C. After incubation one half of each of the resulting cultures was poured into a single flask and well mixed together. The mixture was then distributed into sterile cotton-wool plugged 6 in. by $\frac{3}{4}$ in. or $\frac{5}{8}$ in. tubes in quantities of 3 to 5 c.c., and then about 10 c.c. of fresh peptone water added. Following this, 1 to 2 c.c. of a 6-hour peptone water culture of each strain of *Ps. pyocyanea* against which an active bacteriophage was being sought was added to a separate tube of mixture, which was suitably labelled. The tubes were then incubated overnight at 37° C.

The following day 1 c.c. of the contents of each tube was tipped into a fresh tube, and about 10 c.c. of peptone water was added. The new tube was heated at 57° C. for half an hour which killed the organisms, but did not eliminate bacteriophages (Dickinson, 1947; personal communication), if present. When cool, each tube was re-inoculated with the same strain of *Ps. pyocyanea* as had been added to the mixture on the previous day. The tubes were incubated overnight. This process was repeated as many times as was considered necessary. Two or three times were found to be sufficient. The theory underlying this was that a bacteriophage, if present in the original mixture, and active against the strain inoculated daily, would grow in the sensitive organism and thus increase in titre and specificity for the inoculated strain. The bacteriophage would, in fact, be passaged on a sensitive host organism. As a result of this the preparation would, when tested against the inoculated strain, produce a reaction as sharp and as clear cut as possible. In practice this was found to be the case.

On the day that such preparations were to be tested for the presence of bacteriophage, the tubes and the whole of their contents, which had been incubated overnight, were heated at 57° C. for half an hour. When cool, loopfuls of each preparation were placed on specially-prepared nutrient agar (1 per cent) plates, inoculated with the organism under test for sensitivity, which was the one with which the preparation had been inoculated every day. The plates were prepared in the following way: To 5 c.c. of warm (about 50° C.) molten agar, 5 drops were added from a Pasteur pipette of an overnight broth culture of the strain of *Ps. pyocyanea* to be tested for sensitivity to bacteriophages. The organisms were mixed well with the agar and then poured on the surface of a nutrient agar (1 per cent) plate (9 cm. in diameter) which had been poured on the previous day and dried in the incubator at 37° C. overnight. This procedure was designed to produce a layer of agar about 1 mm. thick seeded with organisms of the strain under test. From this time onwards, until the plate was put in the incubator last thing at night, the lid was propped slightly open so that excess water did not condense on the surface of the seeded agar. With the lid open, then, the plate was dried in the incubator for 1 hour, after which a loopful (2 to 4 mm. in diameter) of each of the appropriate bacteriophage preparations were placed on the surface of the seeded agar in areas suitably delineated by grease pencil on the bottom of the dish. Then it was left at room temperature until last thing at night, when the lid was completely closed for the first time. The plate was placed in the incubator, incubated overnight at 37° C. and examined for bacteriophage activity the following morning.

Method of passaging bacteriophages on sensitive hosts.

This method has been used by many workers, including Craigie and Yen (1938). When bacteriophages were detected by the method described above, about 1 c.c. of the heated preparations which had been shown to contain bacteriophages was tipped into a fresh test-tube and 10 c.c. of peptone water was added, and the resulting mixture was inoculated with the sensitive organism. This was incubated at 37° C. overnight and then about 1 c.c. tipped into a fresh tube, peptone water added, heated at 57° C. for half an hour and re-inoculated with the same strain, as has been described above. As the bacteriophages in the preparation had, in fact, been passaged on the sensitive organisms in the process of detection they were only passaged two more times.

Method of preparing a bacteria-free preparation of bacteriophage.

Bacteriophage preparations, prepared as described in the above section, when fully potent, were centrifuged for 20 minutes at 3500 revolutions per minute in an angle centrifuge. The supernatant fluid was transferred immediately to a sterile test-tube and heated at 57° C. for half an hour. This was then stored in the refrigerator, and tested for potency and the presence of bacteriophage plaques in dilutions as will be described in the next section.

Titration of bacteriophage preparations.

Serial tenfold dilutions of cell-free bacteriophage preparations were made. Wright's (1923) technique of serial dilution was used, in which measured drops of sterile distilled water were placed on a sterile slide coated with sterile wax. The serial dilutions were then made in the usual way with the measured drops. As a routine measure the highest dilution reached was 10^{-6}. Occasionally when separate plaques were not obtained in the 10^{-6} dilution the suspension was titrated to a dilution of 10^{-9}. One loopful of each dilution was then placed on an agar plate, seeded with organisms of the strain against which the bacteriophage preparation had been found active. The plate was prepared and treated in exactly the same fashion as described under the heading "Preparation of bacteriophage suspensions." On the following day, after incubation overnight at 37° C. the plate was examined and the following points especially noted:

1. The character of the clearing obtained with undiluted bacteriophage suspension.
2. The presence or absence of bacteriophage plaques in the higher dilutions.
3. The size and other characteristics of bacteriophage plaques if present.
4. The highest dilution in which a bacteriophage reaction occurred.

The technique of plating loopfuls of tenfold dilutions of bacteriophage suspension on an agar plate culture of the sensitive organisms was used by Craigie and Yen (1938) for the determination of the concentration of bacteriophage suspension to be used for typing certain typhoid bacilli. The result of a titration is shown in Fig. 1.

Purification of bacteriophage preparations.

Some preparations obviously contained more than one type of bacteriophage, since some of the plaques obtained with a single bacteriophage preparation, by the method of titration described above, had an appearance different from others. Pure strains of bacteriophage were obtained by touching a single plaque on such a plate with a straight wire, and then agitating the wire in about 1 c.c. of sterile distilled water. Loopfuls of tenfold dilutions of this distilled water preparation were placed on another specially-prepared plate, inoculated with the same strain that had shown the plaque which had been touched with the straight wire; this was incubated and examined the following day. This procedure was repeated twice, but should be repeated until the preparation shows a uniform type of plaque on its sensitive host.

Although bacteriophages of *Ps. pyocyanea* were purified by this method, pure suspensions were not used here for reasons of speed and convenience.

Derived bacteriophage preparations.

A bacteriophage preparation was not considered fully active against a strain unless it produced a confluent clearing on that strain. When a potent bacteriophage which had been passaged on one strain was found to produce only a few plaques on a previously-untested strain, then the preparation was passaged on the latter strain, and when fully potent was called a derived bacteriophage preparation. Even if it retained its full potency for the strain on which it had originally been passaged, it was regarded as a different bacteriophage preparation, because the number of strains against which it was fully active had been increased as compared with the original from which it had been derived. The bacteriophage causing the appearance of plaques on the second strain may either have been one whose activity had been enhanced by passaging it on the first strain or another carried by the first strain. It was deemed unnecessary to determine this point at the time, but it was borne in mind that the derived and the original bacteriophage were not of necessity the same.

Notation.

All the strains of *Ps. pyocyanea* were numbered, but the numbers were not consecutive.

When cultures of various strains were mixed together, each particular mixture was designated by a letter of the alphabet; for instance "L" represents a mixture of approximately equal parts of 6-hour peptone water cultures of Strains No. 139, 140 and 141.

Notation for bacteriophage suspensions.

This is essentially that used by Wilson and Atkinson (1945) for staphylococcal bacteriophages. The number of the strain or the letter representing the group of strains from which a bacteriophage preparation was obtained was written first followed by a stroke (/), after which was written the number of the sensitive organism on which the bacteriophage or mixture of bacteriophages had been passaged. For example, "H/125" means that cultures of the 23 strains, which were mixed together to make the H group of strains, produced one or more bacteriophages in a preparation which was active against Strain

No. 125, and that the bacteriophages in it had been passaged on Strain No. 125. Initially three bacteriophage preparations were obtained from single strains; in these cases the number of the strain replaced the alphabetical letter preceding the stroke.

When one bacteriophage preparation was derived from another to produce the derived bacteriophage preparations, as described in the last section, then the cypher describing the original preparation is followed by a stroke followed by the number of the new sensitive host strain, thus: H/125/55, which means that the bacteriophage preparation No. 125 produced a few plaques when tested against Strain No. 55 of *Ps. pyocyanea*, and has been subsequently passaged on that strain to produce a potent bacteriophage preparation which produces a confluent clearing on Strain No. 55.

Notation for the different types of bacteriophage reaction observed.

These fall under two headings:

1. Confluent clearings—as obtained with undiluted potent bacteriophage preparations.
2. Separate plaques.

1. *Confluent clearings.*

C++++ denotes a crystal clear confluent clearing (Fig. 2, *a*).
C+++ denotes slightly opaque (Fig. 2, *b*).
C++ denoted hazy (Fig. 3, *a*).

The following symbols were appended to those denoting the degree of clearing:

M denotes a confluent disc of growth of organisms occupying the central portion of a clearing, leaving a margin of complete clearing (Fig. 4, *a*; 5).

H denotes a halo around the clearing produced by the bacteriophage preparation (Fig. 6).

S denotes scattered colonies lying in the area of clearing (Fig. 6).

2. *Separate plaques.*

P++++ denotes plaques so numerous that they are practically confluent (Fig. 3, *c*).

P+++ denotes diminishing numbers of separate plaques (Fig. 2, *c*, *d*; 3, *b*; 4, *b*, *c*).

P++ denotes ditto.

P+ ditto.

Notation applied to the results of titrations.

Table I shows the results of three typical titrations.

TABLE I.—*Dilutions of Bacteriophage Preparations.*

	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1	C++++	C++++	C+++	P++++	P+++	P++	P+
2	C+++	C+++	P+++	P+++±	P+	—	—
3	C+++	C+++	C+++±	C++++	P++++	P+++	P+++
	M	M					

TABLE II.—*The Properties of Some Bacteriophages of* Ps. pyocyanea.

Number of bacteriophage preparation.	Identification of bacteriophage preparation.	Result of titration.	Appearance of confluent clearing.	Size and appearances of plaques.	Susceptibility to Seitz filtration.	Susceptibility to broth.	Clearing in a fluid medium.
1	1/3/46	C++++−P++ 10^{-6}	Crystal clear	Large and medium, clear	−	−	+
2	30/3	C++++−P++++ 10^{-9} HS	Crystal clear scattered resistant colonies	Medium, clear	−	−	+
3	B/29	C+++−P+ 10^{-6}	Fairly clear	Medium and small, clear	−	−	..
4	C/29/60	C++−P+ 10^{-6}	Hazy	Medium, hazy	..	−	..
5	E/102/115	C+++−P+ 10^{-5}	Fairly clear	Large and medium, clear	..	−	..
6	E/6	C++−P+ 10^{-6}	Hazy	Medium, hazy	+	−	..
7	53/1/131	C++++−P+++ 10^{-6} S	Crystal clear scattered resistant colonies	Large and small clear	..	−	..
8	H/4	C+++−P+++ 10^{-6}	Confluent resistant growth. Marginal clearing	Large, central papilla	..	−	..
9	H/122	C++−P++ 10^{-6}	Hazy	Medium, hazy	..	−	..
10	H/138	C++−P+ 10^{-5}	Covered with iridescence	Covered with iridescence, small, indistinct	..	+	..
11	H/132	C++−P++ 10^{-6}	Covered with iridescence	Covered with iridescence, small, indistinct	..	−	..
12	H/132/14	C+++−P++ 10^{-6}	Fairly clear	Small, clear	..	+	+
13	H/125	C+++−P+ 10^{-6}	Nearly covered with iridescence. Fairly clear	Small, clear	..	+	..

14	H/125/55	C+++^{S}−P+ 10^{-6}	Fairly clear scattered resistant colonies	Small, clear	..	..	..	..	..
15	H/30	C+++−P++ 10^{-6}	Nearly covered with iridescence. Indistinct	Nearly obscured by iridescence, indistinct	..	..	..	..	−
16	H/22	C+++^{M}−P+ 10^{-6}	Confluent resistant growth, marginal clearing	Medium, hazy	..	..	..	..	..
17	L/91	C++−P++ 10^{-6}	Fairly clear	Small, fairly clear	..	..	..	..	−
18	L/129	C+++^{M}−P+++ 10^{-6}	Confluent resistant growth. Marginal clearing	Medium, clear	..	..	..	..	−
19	L/112	C++++−P+ 10^{-6}	Crystal clear	Small, very small, clear	..	..	..	..	−
20	L/109	C++++^{S}−P+ 10^{-6}	Crystal clear, scattered resistant colonies	Large, medium, clear	..	..	..	..	+
21	L/54	C++++^{HS}−P+ 10^{-9}	Crystal clear scattered resistant colonies	Medium, small, clear	..	..	..	..	..
22	H/132/16	C+++−P+ 10^{-6}	Fairly clear	Small, clear	..	..	+	.	..
23	53/1	C++−P+++ 10^{-6}	Hazy	Medium, hazy	−	−	−	.	..
24	A/2	..	Fairly clear	Not recorded	+	.	−	.	..
25	C/29/115	..	Fairly clear	Not recorded	+	.	−	.	..
26	A/2/7	..	Fairly clear	Not recorded	+	.	−	.	..

+ = Susceptible (6th and 7th column) or the presence of clearing (8th column).
− = Insusceptible (6th and 7th column) or absence of clearing (8th column).
.. = Not examined.

Titration 1 shows a crystal-clear confluent clearing in the two highest concentrations (10^0 and 10^{-1}), gradually becoming less and less in the higher dilutions, a few plaques only being produced in the 10^{-6} dilution. This was written as follows: C++++ — P + 10^{-6}. The third titration, however, shows that the undiluted preparation produces a confluent growth of resistant organisms in the centre of the first clearing, but in a 10^{-3} dilution gives a crystal-clear reaction which gradually diminishes in higher dilutions. This was written $\frac{\text{C}+++}{\text{M}}$ — C++++10^{-3} — P+++10^{-6}. The reason for adopting this method of notation is twofold, firstly because it is descriptive, and secondly it does not suggest an accuracy, implied by a numerical notation, which does not, in fact, exist.

EXPLANATION OF PLATES.

Fig. 1 to 6 show titrations of bacteriophage preparations on sensitive strain of *Ps. pyocyanea*. The dilution fall in tenfold steps from 10^0 to 10^{-6} in an anti-clockwise direction as indicated in each figure.

FIG. 1.—The result of a typical titration as described in the text (Bacteriophage preparation L/112 on a culture of Strain No. 112.)

FIG. 2.—(*a*) Crystal clear clearing.
(*b*) Fairly clear clearing.
(*c*) Plaques (P++).
(*d*) Plaques (P+).
(Bacteriophage preparation L/54 on a culture of Strain No. 54, see No. 21, Table II.)

FIG. 3.—(*a*) Hazy clearing.
(*b*) Plaques (P+++).
(*c*) Plaques (P++++).
(Bacteriophage preparation E/6 on a culture of strain No. 6, see Table II.)

FIG. 4.—(*a*) Marginal clearing.
(*b*) Plaques (P++).
(*c*) Plaques (P+).
(Bacteriophage preparation H/4 on a culture of Strain No. 4, see No. 8, Table II.)

FIG. 5.—Marginal type of clearing shown in the 10^0 dilution. (Bacteriophage preparation L/129 on a culture of Strain No. 129, see No. 2, Table II.)

FIG. 6.—Halo and scattered central colonies in 10^0 dilution. (Bacteriophage preparation 30/3 on a culture of Strain No. 3.)

Confluent clearing appearances.

Fig. 7, 8 and 9 are enlargements of about five times the natural size of various confluent clearings.

FIG. 7.—This shows a halo and scattered central colonies. It is the 10^{-1} dilution of titration shown in Fig. 6.

FIG. 8.—This shows what appears to be a crystal clear reaction to the naked eye. It is the 10^0 dilution of the titration shown in Fig. 2.

FIG. 9.—An example of marginal clearing. The 10^0 dilution of the titration shown in Fig. 5.

FIG. 10.—This figure shows the result of titrating bacteriophage preparation H/132 (No. 11, Table II) against Strain No. 132, and shows the effect of iridescence in obscuring bacteriophage reactions. Plaques often could not be seen in higher dilutions without a hand lens.

Plaque appearances.

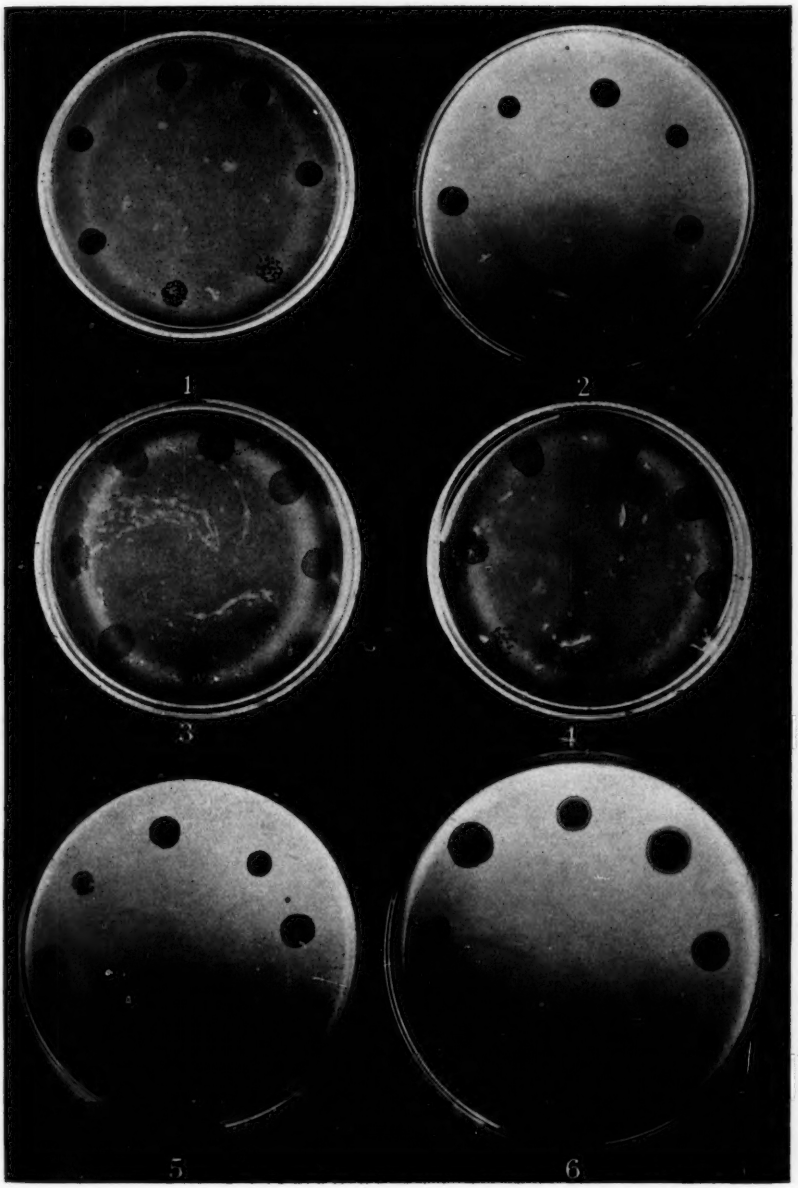
Fig. 11,12, 13 and 14 are enlargements of about five times natural size of various types of plaque.

FIG. 11.—Large and small plaques. This is the 10^{-6} dilution of a titration of 53/1/131 on a culture of Strain No. 131.

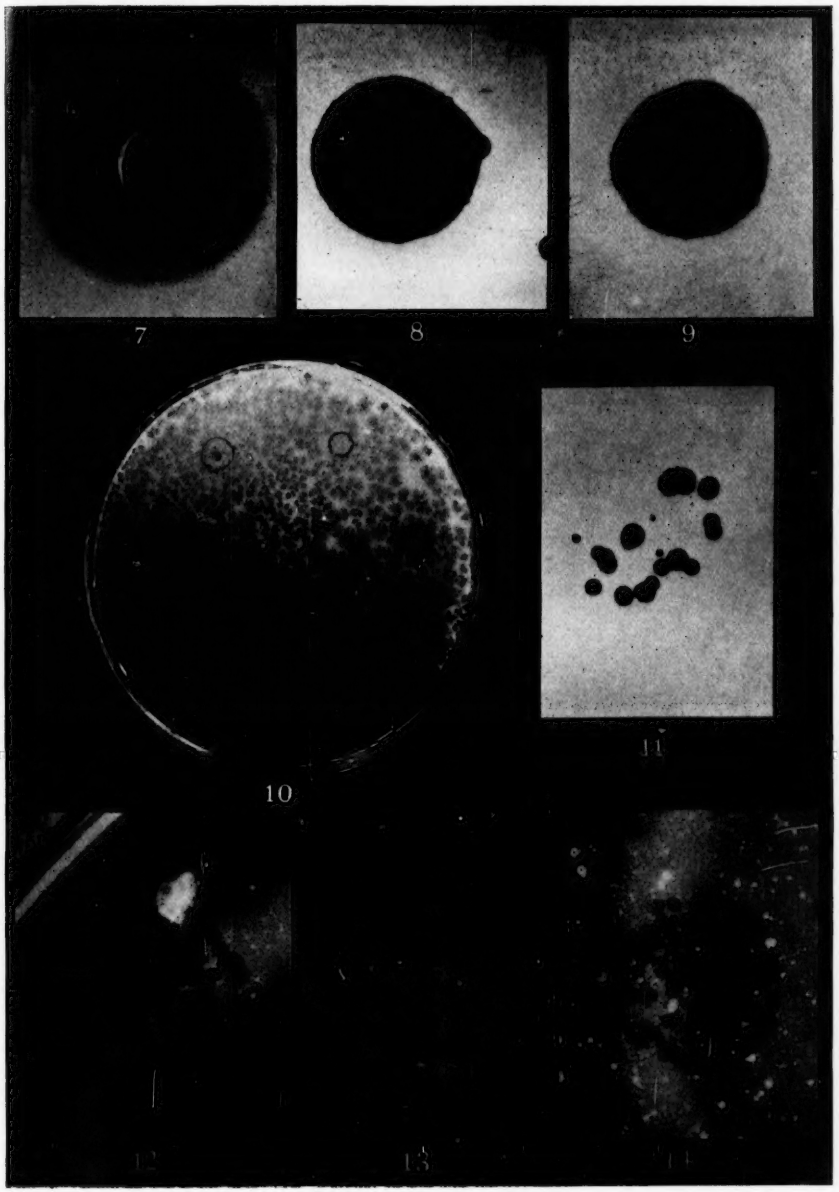
FIG. 12.—Large plaques with central papillae. 10^{-5} dilution of titration shown in Fig. 4.

FIG. 13.—Small and medium-sized plaques. 10^{-6} dilution of Fig. 2.

FIG. 14.—Hazy plaques. 10^{-6} dilution of Fig. 3.



Warner.



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RESULTS.

Altogether 37 bacteria-free fluids have been obtained which, at one time or another, have produced plaques on suitable sensitive strains of *Ps. pyocyanea*. The first twelve were obtained, by methods other than that described in detail, over a period of ten weeks. Twenty-five were obtained over a period of one month by the method described.

The result of a preliminary test for the presence of bacteriophages by the described method was invariably a confluent clearing when bacteriophages were present in the original mixtures, and provided that they were passaged and then tested on a sensitive host strain.

The properties of 26 of the bacteriophage preparations have been sufficiently investigated to merit inclusion in Table II; the remaining ten were found to have the same range of action as one or other of the preparations whose properties are tabulated, and have no other characteristics worthy of note and were therefore set aside.

No. 1–19 in Table II are preparations each with a different range of full action, that is to say, each has produced a confluent clearing on at least one strain of *Ps. pyocyanea* that the others have not, and therefore each has a different bacteriophage content. Twelve of these (No. 8–19) were obtained by the method described in detail. The properties of the remaining seven (No. 20–26) bacteriophage preparations are shown, either because they happen to have been maintained and studied, or because they show points of interest which are to be discussed.

Results of titration.

The third column in Table II shows the results of titration in the notation described. Bacteriophage preparations No. 1–19 only will be considered in this connection as they were studied most closely, and are known to contain different bacteriophages and will, therefore, give a better idea of the incidence of the various types of results. Fifteen show plaques in the 10^{-6} dilution. One (No. 2) showed complete clearing in the 10^{-6} dilution when originally titrated, but when titrated further, showed plaques in the 10^{-9} dilution. Three (No. 5, 10, 14) showed plaques in the 10^{-4} dilution or 10^{-5} dilution. All these bacteriophage preparations showed fairly consistent results when this method of titration was repeated. They were, however, only titrated on two or three occasions each.

The confluent clearing appearances.

The confluent clearing appearances were, apparently, subject to some variation. Whether this is due to minor degrees of variation, either in the organism, or in the degree of moisture of the plate surface, is not known. The results produced by preparations No. 1–19 on their sensitive host organisms are shown in the fourth column, were all obtained over the course of one week, and special care was taken to standardize the cultural conditions as far as possible. They were observed against a black background in a strong light, this being the best method of observing all bacteriophage reactions with *Ps. pyocyanea*.

Four of these preparations show crystal clear reactions; of these No. 2 and 7 have scattered colonies within the area of clearing (Fig. 7); No. 1 and 19 showed no evidence of any such colonies (Fig. 8). Preparation No. 2 also showed a

peculiar halo (Fig. 8), which seemed to be a change in the main confluent growth around the clearing rather than part of it, for the area covered by the halo was not depressed below the surface of the main growth, but was merely lighter in colour, whereas the area of clearing was at a lower level. The halo, as shown in Fig. 7, an enlargement from Fig. 6, has a granular appearance suggesting some inhibition of the growth of the organism.

Preparations No. 8, 16 and 18 showed a confluent growth of organisms resembling a disc lying in the area of clearing giving the appearance of marginal clearing only (Fig. 9). Often this disc of growth was whiter in appearance than the main part of the culture, but not in every case.

Preparations No. 3, 5, 17 and 14 produced a fairly clear confluent clearing. The last of these also showed scattered resistant colonies in the area of clearing.

Preparations No. 4, 6 and 9 showed hazy clearing (Fig. 3*a*).

Preparations No. 10, 11, 13 and 15 were covered in some degree by the confluent, or practically confluent, iridescence produced by their sensitive host organisms, which to some extent obscured the result (Fig. 10).

Thus, it is seen that the different types of reactions obtained are fairly evenly distributed among the different preparations described, and that confluent iridescence interferes with the easy visibility of reactions. This latter phenomenon occurred to a degree sufficient to obscure bacteriophage action partially in 17 strains of *Ps. pyocyanea* at one time or another. A further 16 strains showed a certain amount of iridescence.

The growth occurring within the confluent clearing has been shown to consist of a growth of organisms resistant to the action of the bacteriophage preparations producing the clearings. This is in accordance with the findings with bacteriophages active against organisms other than *Ps. pyocyanea*. However, it occurs more frequently and profusely with the preparations described here than has been seen with bacteriophages specific for coliform organisms.

Plaque appearances.

Plaque appearances, shown in the fourth column of Table II, are, in contradistinction to confluent clearings, more consistent. The size of the plaques produced by a single preparation varied from time to time, but in the main their size relative to that of plaques produced by other preparations, at the same time and under the same conditions, remained fairly constant. This variation in size of plaques was probably due to minor variations in the amount of moisture on the surface of the plate. Other plaque characteristics were consistent.

Again preparations No. 1 to 19 will be considered. Preparations No. 1, 5, 7 and 8 produce large plaques (Fig. 11). Those of No. 5 and 7 also produced some small or medium plaques as well. Preparation No. 8 showed plaques with a central papilla (Fig. 12) of bacterial growth, No. 1, 5 and 7 were clear.

Preparations No. 10, 11, 13 and 15 were covered with iridescence and the plaques were difficult to see, but could be observed, with difficulty, against a dark background with a hand lens. Under these conditions they all appeared small and indistinct. The remainder of the preparations showed either medium sized (Fig. 13) or small plaques (Fig. 13), and they were clear except in the cases of preparations No. 4, 6, 9 and 16, which were hazy (Fig. 14).

The properties of preparations No. 20 to 26 which were examined are similar to those described above.

Susceptibility to Seitz filtration.

Fairly early in this investigation preparations No. 6, 24, 25 and 26 were found to have lost all or nearly all their potency after being passed through a Seitz filter as shown in the sixth column of Table III. It was owing to this and the fact that the above preparations could be obtained successfully when heating at 57° C. for half an hour was substituted for filtration that the latter procedure was abandoned.

TABLE III.—*The Comparison of the Effect of Broth and Peptone Water as Culture Medium on the Propagation of Certain Bacteriophages of* Ps. pyocyanea.

	Bacteriophage identification.	Dilution of bacteriophage preparation.						
		10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Broth as culture medium	H/125	—	—	—	—	—	—	—
	H/138	—	—	—	—	—	—	—
Peptone water as culture medium	H/125	C+++	C+++	P++++	P+++	P++	P+	P+
	H/138	C++	C++	C+	P++	P+	P+	—

Table showing the result of titrating bacteriophage preparations H/125 (Table I, No. 13) and H/138 (Table I, No. 10) obtained by using broth and peptone water as culture media in parallel.

Susceptibility to broth.

When the "H" collection of strains were grown in broth and rendered bacteria-free and then tested against Strains No. 125 and 138 of *Ps. pyocyanea* a few plaques were obtained. After passaging the preparations on the two strains and testing them again, neither the expected confluent reaction nor even a few plaques were obtained. Two new preparations, made in exactly the same way from the beginning, with the exception that peptone water was used instead of broth as a culture medium, were found to be fully active against the two strains (Table II, No. 10 and 13).

As a result of these observations a controlled experiment was carried out, the results of which are shown in Table III, in which the same preparations were produced using peptone water and broth in parallel. Neither broth preparation had any activity, whereas both peptone water preparations produced a confluent reaction.

Susceptibility to broth is shown in Table II in the 7th column. Two other bacteriophage preparations (No. 12 and 22), it will be noted, also showed susceptibility.

Clearing in a fluid medium.

Presence or absence of clearing of fluid cultures of sensitive strains of *Ps. pyocyanea* by bacteriophage preparations was noted in nine of the preparations shown in Table III. In five, No. 1, 2, 12, 20 and 23, it was present. Tenfold dilutions of preparations No. 1, 2, 23 in broth inoculated with sensitive host strains were observed, and it was noted that after incubation overnight the first one or two dilutions were not clear, whereas the third to the eighth or ninth were clear. This phenomenon has been noted in the case of coliform bacteriophages, where it has been shown that the opacity present in the higher concentrations is resistant or secondary growth. Preparations No. 12 and 20 produced marked clearing of their sensitive hosts in fluid culture in an approximately one-in-ten

dilution, which was the only one examined. Preparations No. 15, 17, 18 and 19 did not produce clearing visible to the naked eye in a 1 in 10 dilution, the only one examined.

At the same time as these observations were made it was noticed in broth cultures of certain strains of *Ps. pyocyanea* that growth occured only on the surface of the fluid in the form of a cohesive pellicle, and apparently none occurred in the depth of the fluid. After observing this, clearing of fluid cultures of sensitive strains of *Ps. pyocyanea* by bacteriophage preparations was not recorded.

The Effect of Storage on the Bacteriophage Preparations of Ps. pyocyanea.

The bacteriophage preparations, kept in screw-capped containers or in sterile plugged test-tubes, all maintained their potency during the period of the investigation (Table IV), except one (Table II, No. 23) which suddenly lost its

TABLE IV.—*The Effect of Storage on Bacteriophage Preparations of* Ps. pyocyanea.

Number of weeks stored.	Potency of preparations. Potent.	Not potent.
16	1	0
14	1	0
12	2	1
10	1	0
8	3	0
6	5	0
5	9	0
4	1	0
Total	23	1
Grand Total	24	

This table shows the results of storing the bacteriophage preparations, described in the text, in the refrigerator; each was titrated when first rendered bacteria free and placed in the refrigerator, and then again after a varying number of weeks (Column 1).

activity for its sensitive host strain. Since, however, this bacteriophage preparation had not lost its activity for another sensitive strain, on which it produced a confluent reaction, and since at the same time there was a change in the cultural appearance of the host organism, this apparent loss of potency was thought to be due to the variation of the sensitive host strain. This would be in accord with the findings of Hadley (1924) and others, who found *Ps. pyocyanea* to be prone to spontaneous variation in artificial culture.

Table IV shows that 23 out of 24 maintained their potency for at least 4 to 16 weeks in the refrigerator. The preparation discussed above which lost its potency at 12 weeks had not done so at 10 weeks.

The Range of Action of the Bacteriophage Preparations.

From this point onwards bacteriophage preparations will not be referred to as active against an organism unless it produced a confluent clearing on that organism.

TABLE V.—*The Confluent Reactions Produced by Nineteen Different Bacteriophage Preparations.*

Bacteriophage preparation number.	Bacteriophage preparation identification.	Sensitive host strains of *Ps. pyocyanea*. 46	3	29	60	115	6	131	4	122	138	132	14	125	55	30	22	91	129	112	Other strains against which bacteriophage is active.	Number of strains against which each bacteriophage is active.
1	1/3/46	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	53	3
2	30/3	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	2, 52	3
3	B/29	−	..	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	—	1
4	C/29/60	..	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	29	2
5	E/102/115	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	52, 110, 119	4
6	E/6	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	—	1
7	53/1/131	..	..	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	3, 8, 62, 100, 126	6
8	H/4	..	−	+	..	..	−	−	+	−	−	−	−	−	−	−	−	−	−	−	—	2
9	H/122	..	..	−	..	−	..	−	+	+	−	−	−	−	−	−	−	−	−	−	—	2
10	H/138	..	..	−	−	−	..	−	..	−	+	−	−	−	−	−	−	−	−	−	—	1
11	H/132	..	−	−	−	..	..	−	−	−	..	+	−	−	−	−	−	−	−	−	15, 109	3
12	H/132/14	..	..	−	−	..	..	−	−	−	..	..	+	−	−	−	−	−	−	−	16	2
13	H/125	..	..	..	−	−	..	..	−	−	..	−	..	+	−	−	−	−	−	−	—	1
14	H/125/55	..	..	−	−	..	..	..	−	−	..	..	..	..	+	−	−	−	−	−	3	2
15	H/30	..	..	+	−	..	..	−	−	−	−	..	..	−	−	+	−	−	−	−	3, 29, 36	5
16	H/22	..	−	−	−	..	..	−	−	−	..	−	..	−	..	−	+	−	−	−	—	1
17	L/91	..	..	−	−	..	..	−	−	−	..	..	..	−	..	−	..	+	−	−	3, 76	3
18	L/129	..	−	−	−	..	..	−	−	−	−	..	−	−	−	−	..	−	+	−	—	1
19	L/112	..	..	−	−	..	..	..	+	−	..	..	..	−	−	..	..	..	..	+	—	2
Number of bacteriophages fully active against each strain		1	2	3	1	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1		

\+ = confluent clearing.
− = no action or a few plaques.
.. = not examined.

NUMBER OF DIFFERENT STRAINS OF *Ps. pyocyanea* ON WHICH A CONFLUENT REACTION HAS BEEN OBTAINED = 35.

The table is a summary of the confluent clearings produced on sensitive host organisms by 19 different bacteriophage preparations (see Table II, No. 1–19) obtained during the course of isolating bacteriophages from lysogenic strains. (Not all 65 strains were examined by all bacteriophage preparations.)

Bacteriophage preparations are arranged in chronological order of isolation from top to bottom. Sensitive host strains are arranged in the same way from left to right.

The preparations No. 1 to 19 were said each to have a different range of action, meaning that each produced a confluent clearing on at least one strain on which the other preparations did not. Table V shows the reactions obtained by preparations No. 1 to 19 on their sensitive host organism. It is so arranged that the bacteriophage preparations are in the chronological order in which they were isolated from above downwards, and their sensitive hosts are in the same order from left to right. By this arrangement the positive signs, representing confluent reactions with sensitive host organisms, are disposed in a diagonal line running across the table. If the top two transverse lines are disregarded for the moment, it will be noted that all the symbols to the right of the diagonal line are negative signs. Thus, all the positive signs (except those in the top two transverse lines) have negative signs above them. This signifies that a bacteriophage preparation, in the same transverse line as a particular positive sign, is the first one isolated to act on the organism at the head of the column, in which the positive sign lies, thus indicating that each fresh bacteriophage preparation has a range of action different from previous ones and, therefore, a different bacteriophage content. This was ensured by the system adopted for dealing with strains of *Ps. pyocyanea* when testing for lysogenicity and for sensitivity to bacteriophage preparations, which was described above. Had this system been adhered to slavishly there would have been nothing but dots, representing " not tested," on the left-hand side of the diagonal of positive signs, as each strain, when a bacteriophage preparation was isolated active against it, was put on one side and not used in further tests for sensitivity to bacteriophage preparations isolated at a later date. However, in other connections, these organisms were tested with such preparations and the results are recorded. The two bacteriophage preparations at the top of the table both isolated together are obviously different, because the first is active against the first two organisms and the second only against the second organism.

On the right-hand side of the table is a column showing strains, other than the sensitive hosts, which are also sensitive to various bacteriophages.

Table V also shows that at least 35 of the 65 strains were sensitive to one or other of the 19 bacteriophage preparations, which if a representative sample of strains of *Ps. pyocyanea* were examined here, suggests that about 54 per cent of strains could be typed with these bacteriophages.

In addition Table V shows that 6 bacteriophage preparations were active against only one strain of *Ps. pyocyanea* each. Six were active against two strains, four were active against three, one against four, one against five and one against six. This suggested that strains of *Ps. pyocyanea*, susceptible to bacteriophages, would fall into groups if typed with the preparations in hand.

Finally, Table V shows that on 16 strains of *Ps. pyocyanea* a confluent clearing is produced by only one bacteriophage preparation, on Strain No. 3 two bacteriophage preparations produced a clearing, and on Strain No. 29 and No. 4 three do so. However, since not all strains were tested with all bacteriophage preparations, no conclusions can be drawn as to the types into which strains of *Ps. pyocyanea* used here would fall.

DISCUSSION.

The differences between the method of isolating the bacteriophages of *Ps. pyocyanea*, described here, and those used by previous workers for bacterio-

phages active against other organisms, are four in number. Firstly, instead of testing one strain of organism at a time for lysogenicity, cultures of several strains were bulked together and then tested. Secondly, at no stage was filtration used. Thirdly, peptone water was used as a culture medium instead of broth, and finally, agar plates used for testing for bacteriophage activity were inoculated with a layer of agar and seeded with organisms, instead of applying a fluid culture to the surface.

The method of bulking cultures of strains of *Ps. pyocyanea* and testing the resultant mixture is likely to yield a larger number of bacteriophages, and permits a greater number of strains to be examined in any one period of time than with classical methods. However, it should be borne in mind that some bacteriophages, when present in the same environment, can interfere with the development of others (Delbrück and Luria, 1943), and thus perhaps not all the bacteriophages present in a mixture of a number of strains of *Ps. pyocyanea* will be propagated. In addition, the possibility exists that some strains of *Ps. pyocyanea* produce substances which inhibit the growth of others, thereby preventing the multiplication of bacteriophages active against inhibited strains. In spite of these objections, this method has apparently been efficient enough to isolate bacteriophages with a fairly wide range of action.

The continued use of Seitz filters to obtain fluids free from living bacteria and of broth as a culture medium would have diminished the yield of bacteriophages derived from lysogenic strains of *Ps. pyocyanea*. Both were abandoned because more efficient substitutes, to wit, centrifuging and heating at 57° C. for half an hour or heating alone and peptone water replaced Seitz filters and broth respectively.

In the early stages of this investigation, while using more conventional methods of detecting bacteriophages, progress was difficult and slow. The difficulty was partly to be attributed to the profuse growth of *Ps. pyocyanea* when inoculated on the surface of an agar plate. The tendency of this organism to spread and its luxuriance of growth obscured some bacteriophage reactions and made others less clear cut. Seeding organisms in a thin layer of agar on the top of an agar plate checked excessive growth. The difficulty was also attributed to the fact that owing to the nature of these particular bacterium-bacteriophage reactions, even some of the potent bacteriophage preparations described (see preparations No. 4, 6, 9, 10, 11, 13 and 15, Table II) produced reactions which were relatively faint or difficult to see on account of confluent iridescence. The method whereby bacteriophages, when present in mixtures of cultures of various strains, were passaged on strains sensitive to them, before actually testing for their presence, ensured as clear cut a reaction as was possible in each case when, finally, they were tested against the sensitive strain on which they had been passaged.

Besides being comparatively simple, the method used showed its value by the fact that 25 bacteriophage preparations were obtained, by its use, in 4 weeks, while only 12 were obtained by the other methods in 10 weeks.

The technique of titration of bacteriophage preparations was chosen because it was simple, required no special apparatus and used a minimum quantity of bacteriophage preparations. It also had the following three useful attributes: Firstly, it gave an approximate estimate of potency; secondly, the morphology

of the confluent clearings and plaques could be examined, and at the same time it could be seen whether the preparation was suitable for bacteriophage typing; and thirdly, the separate plaques obtained could be used for the purification of the different bacteriophage strains.

The most noticeable characteristic of the bacteriophages of *Ps. pyocyanea* was the readiness with which resistant growth appeared in the centre of the confluent clearings. It is believed to be this factor which made the reactions of this group, as a whole, less clear cut than those bacteriophages specific for other organisms. The rapid and profuse production of variants resistant to bacteriophage is in character with the ease with which this organism undergoes variation, as shown by Hadley (1924), Linz (1938) and others. This latter point is emphasized by the fact that one strain apparently spontaneously lost its sensitivity during this part of the investigation to a bacteriophage preparation that had been passaged on it and had been fully active against it. The strain had been kept on artificial culture media for several months. Another point of difference from other races of bacteriophages is that those of *Ps. pyocyanea* after being passaged do not reach a very high titre or degree of potency. In other respects, so far as is known, the bacteriophages of *Ps. pyocyanea* are typical of bacteriophage as a whole.

One interesting side issue is that some bacteriophages cannot be isolated from lysogenic strains of *Ps. pyocyanea* when grown in broth, but can when in peptone water (Table III). Since the broth used here was found to contain less ionic calcium than the peptone water, and since Bordet and Bordet (1946) have shown that an optimum concentration of these ions is necessary for the clearing of a broth culture of *Sh. shiga* by bacteriophage, experiments have been performed using synthetic media to try and determine this point. The results so far have shown that these bacteriophages can be isolated in a medium completely free of calcium ions. This example shows a possibly profitable line of research into the host-virus relationship, especially so because, as mentioned in the introduction, *Ps. pyocyanea* grows readily in very simple synthetic media (Robinson, 1932).

The adequate keeping qualities of the 19 bacteriophage preparations (Table IV), which have been described, their fairly wide range of action (Table V) and the large proportion of organisms sensitive to them (Table V) suggest that the bacteriophages of *Ps. pyocyanea* could be suitably applied to bacteriophage typing of strains of that organism. This is supported by the fact that strains No. 23 and 29 (Table V) were isolated from a patient mentioned by Buxton, Simon and Selbie (1949) on two occasions, separated by a fortnight, and confluent clearings were produced on both by bacteriophage preparations No. 3 and 15. In addition organisms No. 76 and 91 (Table V) were isolated from the inflamed eyes of two new-born children in the same ward at the same time. A confluent clearing is produced on them both by bacteriophage preparation No. 17. The result of this last example suggests that either cross-infection or infection from a common source had occurred. The result of the first example may not be altogether surprising, but it does demonstrate that bacteriophage typing of strains of *Ps. pyocyanea* can be successfully performed. There are, however, two difficulties: firstly, that bacteriophage reactions can be obscured by the confluent iridescence that is produced by a moderately large proportion of strains of *Ps. pyocyanea*, and secondly, that if the sensitive host organisms vary, as is thought

here, so that bacteriophage cannot be passaged on them, then perhaps useful preparations may be lost.

Finally, if the preparations in hand either have not a sufficiently wide range of action or are not all suitable in any other way, it would not be difficult to isolate more bacteriophages by the methods described, which could replace or could be added to the present stock.

SUMMARY.

Sixty-eight strains of *Ps. pyocyanea*, showing the usual staining, cultural and biochemical characteristics, mostly isolated from hospital patients, were used in this investigation.

A simple method of isolating bacteriophages from lysogenic strains of *Ps. pyocyanea* is described. Broth as a culture medium and Seitz filtration had a deleterious effect on some bacteriophages. Filtration was abandoned in favour of centrifuging and heating or heating alone. The methods of titration, purification and notation, used here, are described.

The properties of 26 of these bacteriophage preparations are tabulated. Nineteen of the preparations are dealt with in more detail, because each was active against a different range of strains of *Ps. pyocyanea* and therefore had a different bacteriophage content. The confluent clearing and plaque appearances produced by these preparations are described in detail. The bacteriophage preparations produced confluent clearings on at least 35 (54 per cent) out of a total of 65 strains of *Ps. pyocyanea*.

With one apparent exception all bacteriophage preparations were shown to have maintained their potency for at least from four to sixteen weeks in the refrigerator.

The conclusion is that it is possible to separate strains of *Ps. pyocyanea* into groups according to the bacteriophages which act upon them.

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